Simultaneous measurements of Ca^{2+} in the intracellular stores and the cytosol of hepatocytes during hormone-induced Ca^{2+} oscillations

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Abstract Simultaneous Ca^{2+} measurements in the cytosol and intracellular stores (IS) of rat hepatocytes were performed using two Ca^{2+}-sensitive probes (Fluo-3 and Mag-fura-2), and combined whole-cell patch clamp and fluorescence microscopy. A steady-state Ca^{2+} concentration of ~630 μM was estimated in the IS. α_1-Adrenergic stimulation induced periodic elevations of cytosolic Ca^{2+} and parallel synchronized transient declines in the IS. Subsequent application of the intracellular Ca^{2+}-pump inhibitor thapsigargin resulted in a release of Ca^{2+} from the IS to reach a level of Ca^{2+} depletion much lower than the lowest transient decline observed during the oscillations.

Key words: Intracellular Ca^{2+} store; Ca^{2+} oscillation; α-Agonist; Phenylephrine; Thapsigargin

1. Introduction

Hepatocytes respond to glycogenolytic agonists, such as vasopressin, angiotensin II and α-adrenergic hormones by increasing their cytosolic free Ca^{2+}. At constant agonist concentration, cells studied individually exhibit periodic variations of free Ca^{2+} concentration, referred to as Ca^{2+} oscillations [1,2]. Increasing the agonist concentration leads to a frequency, rather than amplitude, increase of oscillations.

In a previous study from our laboratory [3] we showed, based on experimental and theoretical evidence, that Ca^{2+} oscillations elicited in hepatocytes by phenylephrine, an α_1-adrenergic agonist, could be explained by a simple one-pool model. This model proposed that binding of the hormone to the receptor leads to the production of inositol 1,4,5-tris-phosphate (IP_3) via phosphatidyl inositol bis-phosphate breakdown. The concentration of IP_3 is then supposed to increase in the cytosol and its binding to the IP_3 receptor/Ca^{2+} channel of the intracellular Ca^{2+} stores (IS) will induce the release of Ca^{2+}. This release subsists until a certain threshold cytosolic free Ca^{2+} concentration is reached. After this, the cytosolic Ca^{2+} concentration brought back to its original level by active pumping into the IS and/or out of the cell by Ca^{2+}-ATPases. Although the feedback mechanisms that orchestrate the Ca^{2+} oscillations remain unclear, our model [3] proposed that Ca^{2+} provides positive feedback on its own release from the IP_3-sensitive Ca^{2+} pool. Similar minimal mechanisms for Ca^{2+} oscillations are also assumed in other models [4].

A crucial variable that is largely unknown at this point is the fate of Ca^{2+} in the IS during oscillations. Simultaneous measurements of Ca^{2+} in the IS and in the cytosol should help clarify the uncertainties about the steady-state Ca^{2+} concentration as well as the existence or time course of Ca^{2+} concentration variations in the IS during a train of oscillations.

Previous successful attempts have been made to measure Ca^{2+} in the IS [5,6]. In both cases, the strategy was to load the cells with low affinity Ca^{2+} probes using acetoxymethyl esters (AM) membrane permeant derivatives, and take advantage of the propensity of such dyes to distribute in both cytosol and sub-cellular compartments. In order to eliminate cytosolic dye molecules and detect only the signal originating from the intracellular compartments, Hofer and Machen [5] permeabilized gastric gland cells, a treatment that would render hepatocytes unable to oscillate. Tse et al. [6] used the patch clamp technique in whole-cell mode to perform intracellular dialysis of gonadotropes and measure Ca^{2+}-activated K^+ currents, an indirect measure of cytosolic Ca^{2+}. We chose to pursue a similar approach but, in addition, to directly measure Ca^{2+} in the cytosol by introducing a second fluorescent probe from the patch pipette.

2. Materials and methods

2.1. Preparation of hepatocytes

Hepatocytes were prepared as described in detail previously [7]. Briefly, liver of male Wistar rats was perfused with an oxygenated calcium-free EDTA solution. Mechanical dissociation of liver tissue followed by two sequential centrifugations allowed the isolation of single hepatocytes with a population viability > 80%. Cells were then frozen in fetal bovine serum supplemented with 10–15% DMSO and kept up to 10 days in liquid nitrogen. On experiment day, cells were thawed, washed, deposited on collagen-coated glass coverslips and incubated at 37°C for 2 h in F12-bicarbonate medium containing 2% bovine serum albumin (BSA) in 5% CO_2/95% air atmosphere. Unattached cells were washed out and coverslips were kept at 4°C in F12-HEPES with 1% BSA for a whole day.

2.2. Dye loading procedures

Hepatocytes were loaded for 30 min at 37°C with 4 μM Mag-fura-2 AM and placed in a dye-free solution for an additional 30 min. This procedure was found to maximize the localization of the dye in the intracellular compartments. Dye-loaded cells were then mounted in a superfusion chamber on the stage of an inverted microscope. Experiments were performed at room temperature. As depicted in Fig. 1, cells were patched in whole-cell configuration using a pipette containing Fluo-3 (ionic form), a dye commonly used to measure cytosolic free Ca^{2+}. Mag-fura-2 and Fluo-3 were selected for their K_d for Ca^{2+} (53 μM and 316 nM, respectively) and their compatible spectral characteristics. During the first minutes of intracellular perfusion, cytosolic Mag-fura-2 was washed out while Fluo-3 simultaneously filled the cytosolic compartment. At equilibrium, i.e. about 10 min after establishment of

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Abbreviations: ER, endoplasmic reticulum; IS, intracellular Ca^{2+} stores; AM, acetoxymethyl ester.

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whole-cell patch clamp, Fluo-3 was located in the cytosol while Mag-fura-2 was associated with intracellular compartments (Fig. 2).

2.3. Instrumentation

The setup for simultaneous fluorescence microscopy and patch clamp consisted in an intensified video camera (Videoscope Int., Washington, DC, USA) attached to an inverted microscope (Nikon, Tokyo, Japan) equipped with a 100 × 1.3 N.A. objective lens (Nikon). The video image was digitized by an image processor (Leutron, Glattburg, Switzerland) that allowed the selection of regions of interest and intensity measurement inside single cells. A fluorescence filter wheel (Suter Instr., Novato, CA USA) was used to sequentially illuminate the preparation with 480 (Fluo-3), 340 and 380 nm light (Mag-fura-2). The filter cube contained a 510 nm dichroic mirror and 520 nm longpass emission filter. No significant cross-talk was detected between the two fluorescent dyes. Whole-cell patch clamp was performed using 1–3 MΩ pipettes and voltage clamp amplifier system (Axon Instr., Foster City, CA USA). Pipette and headstage amplifier were positioned using a water hydraulic micromanipulator (Narishige, Tokyo, Japan).

2.4. Solutions and materials

Bath solution was F12 basic culture medium containing 10 mM HEPES, 1.8 mM $CaCl_2$ and set to pH 7.4. Pipette solution contained (mM): 138 K-glutamate, 8 NaCl, 5 $MgCl_2$, 1 EGTA, 0.5 $CaCl_2$, 10 HEPES, 1 ATP, 1 GTP, 2 glutathione, 0.02 Fluo-3 and was adjusted to pH 7.2. Mag-fura-2 AM and Fluo-3 were from Molecular Probes (Eugene, OR, USA). ATP, F12 medium, BSA, thapsigargin and ionomycin were from Sigma (St. Louis, MO, USA). The other chemicals were from Fluka (Buchs, Switzerland).

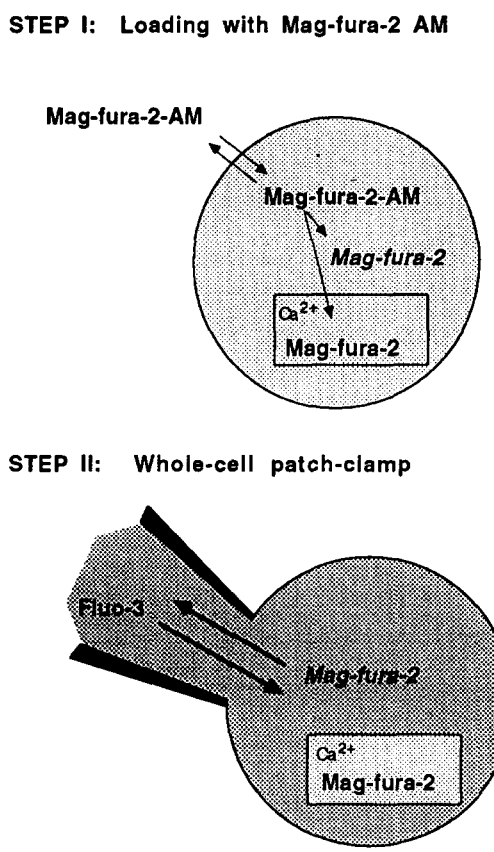

Fig. 1. Scheme of the experimental procedure. In a first step, hepatocytes were loaded with Mag-fura-2 which distributed in both cytosol and sub-cellular compartments. In a second step, the cells were patched in whole-cell configuration. The Ca^{2+}-dye Fluo-3 (anionic form), present in the pipette solution, progressively diffused into the cytosol, while cytosolic Mag-fura-2 was washed out in the pipette. This procedure allowed to keep the Mag-fura-2 trapped in the sub-cellular compartments. At equilibrium, i.e. after about 10 min of whole-cell patch clamp, Fluo-3 was located in the cytosol while Mag-fura-2 remained associated with the intracellular stores.

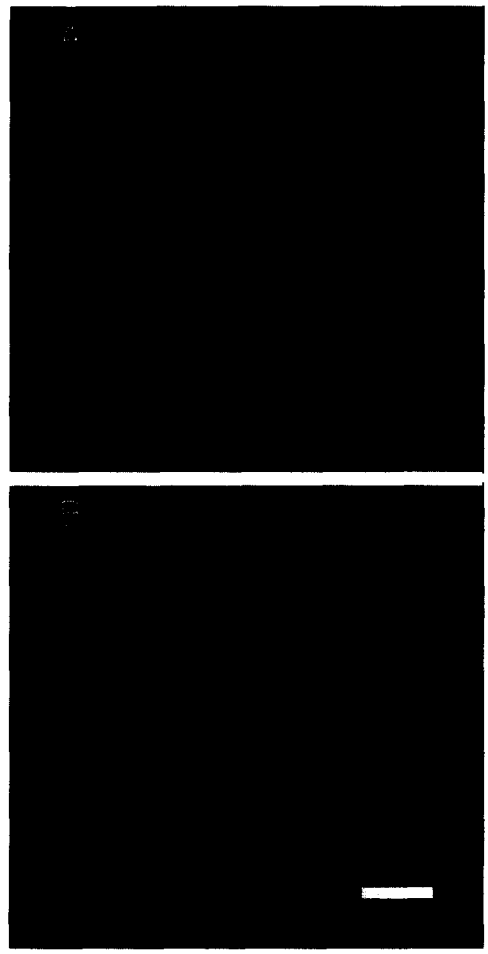

Fig. 2. Digitized fluorescence images of an hepatocyte after dye exchange. Illumination at 480 nm (panel A) and 340 nm (panel B) demonstrates the distribution pattern of Fluo-3 and Mag-fura-2, respectively. Scale bar = 5 μm.

3. Results

Loading cells with AM derivatives of fluorescent dyes often results in the distribution of the dye molecules into sub-cellular compartments (e.g. see [8]). This observation has also been made with our hepatocyte preparation [7], and led us to work out a loading procedure that would result in a maximal distribution of dye in intracellular compartments. Because Ca^{2+} is expected to be high in the IS, we chose to load the cells with Mag-fura-2 AM. This dye possesses a relatively high K_d for Ca^{2+} (53 μM) [9] and had previously been successfully employed for IS Ca^{2+} measurement [5]. Intracellular dialysis was then performed on dye-loaded cells using whole-cell patch clamp techniques (Fig. 1). The patch pipette was filled with a solution containing the Ca^{2+} dye Fluo-3, a dye suitable for cytosolic free Ca^{2+} measurements. After rupture of the patch of membrane simultaneous Mag-fura-2 washout from- and Fluo-3 incorporation into the cytosol occurred. The extent of exchange was monitored by sequential illumination at 480 nm (Fluo-3), 340 and 380 nm (Mag-fura-2). Satisfactory exchange

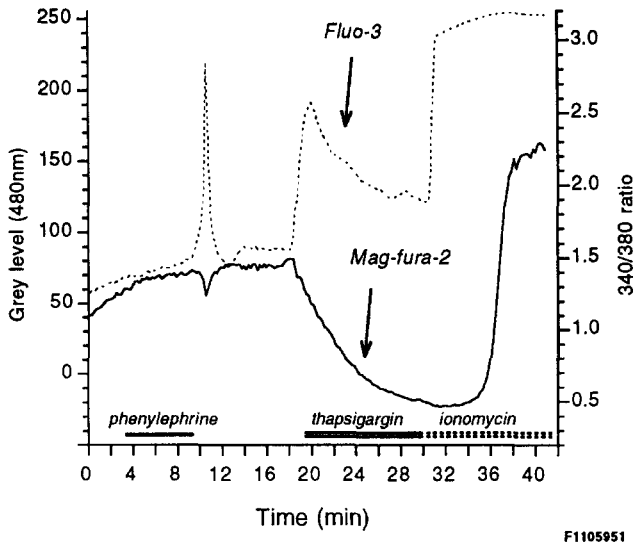

Fig. 3. Measurement of Ca^{2+} in the IS and cytosol. Fluo-3 signal measured at 480 nm (dotted line, left ordinate) and 340/340 nm ratio (solid line, right ordinate) are represented in the graph. Image acquisition was started 9 min after establishment of whole-cell patch clamp when Fluo-3 signal and Mag-fura-2 ratio had both almost reached a plateau value. Additions of phenylephrine (3–5 μM), thapsigargin (1 μM) and ionomycin (4 μM) are indicated in the graph.

was reached about 10 min after establishing the whole-cell configuration with a pipette series resistance in the range 3–8 MΩ. The fluorescence excitation ratio of 340/380 nm was computed and used as an estimate of the Ca^{2+} concentration in the IS.

After establishment of the whole-cell configuration, the 340/380 nm ratio gradually increased, indicating that the dye was progressively sensing a calcium-rich compartment, while the absolute 340 nm and 380 nm signals of Mag-fura-2 diminished by >85%. A similar observation has been made by Hofer and Machen [5] after permeabilization of the cells with digitonin. During that time, Fluo-3 diffused from the pipette into the cell and both signals reached a plateau after about 10–12 min. Fluo-3 generally exhibited a somewhat slower kinetics of diffusion than Mag-fura-2, probably due to its higher molecular weight. Examination of the fluorescent images (Fig. 2) after dye exchange showed that the Mag-fura-2 signal was punctuated and localized in areas surrounding the nucleus, while Fluo-3 remained more diffused in the cell interior. This observation is qualitatively consistent with a predominant distribution of Mag-fura-2 inside intracellular sub-compartments.

Fig. 3 presents experimental traces in which the image acquisition started 9 min after rupture of the patch of membrane. The cell response after application of phenylephrine (3–5 μM) consisted in a transient increase of the Fluo-3 signal accompanied by a simultaneous transient decrease in Mag-fura-2 ratio. When the ER Ca^{2+}-pump inhibitor thapsigargin (1 μM) was subsequently added, the expected Ca^{2+} release from the stores occurred and could be seen both as a decrease of the Mag-fura-2 ratio and a simultaneous transient increase of the Fluo-3 signal. When the stores were almost depleted, ionomycin (4 μM) was added to the Ca^{2+}-containing bathing medium. This maneuver induced a fast Ca^{2+} entry detected in the Fluo-3 signal, and with some delay, in the Mag-fura-2 ratio. This delay was consistent with a slower incorporation of ionomycin in the IS membranes. Similar observations were done in >20 preparations and are good indications that the free Ca^{2+} concentration was measured in the cytosol and IS separately.

From experiments using the same protocol as illustrated in Fig. 3, the Ca^{2+} concentration in the stores was estimated using the equation initially described for the calibration of ratio dyes such as Fura-2 [10]:

$$Ca^{2+} = K_d \times \frac{R - R_{min}}{R_{max} - R} \times \frac{S_{f2}}{S_{b2}}$$

where R is the fluorescence ratio F_{340}/F_{380}, R_{min} and R_{max} are the ratio in zero and saturating Ca^{2+} concentrations, respectively, and K_d is the effective dissociation constant of the dye for Ca^{2+}. S_{f2} and S_{b2} represent the F_{380} signal obtained in zero and saturating Ca^{2+} concentrations, respectively. We used the value after thapsigargin as an approximation of zero Ca^{2+} value, and the value after ionomycin as the saturating level. The K_d was taken as 53 μM [9]. Using this approach, the Ca^{2+} concentration in the IS was estimated to be 628 ± 221 μM (n = 8).

Fig. 4 shows a cell that exhibited Ca^{2+} oscillations when stimulated with 3 μM phenylephrine. While the Fluo-3 signal demonstrated periodic transient elevations of cytosolic free Ca^{2+}, the Mag-fura-2 ratio showed corresponding periodic declines of the Ca^{2+} concentration. The oscillations of Mag-fura-2 and Fluo-3 signals occurred in synchrony, consistent with a direct Ca^{2+} exchange between the IS and the cytosol.

When the oscillations stopped, thapsigargin was added and a further release of Ca^{2+} from the IS into the cytosol was evidenced as a decline of Mag-fura-2 ratio and a transient increase of Fluo-3 signal. The gradual Ca^{2+} depletion of the stores during oscillations, observed in all the oscillating cells, was probably due to the presence of the Ca^{2+} buffer EGTA in the pipette, which represents an infinite sink for Ca^{2+}. These observations, made in 13 different hepatocyte preparations, demonstrated that the IS was only partially depleted of Ca^{2+} during an oscillatory transient.

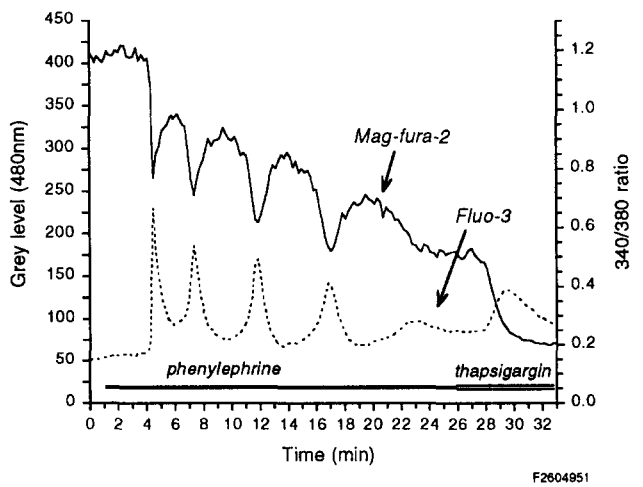

Fig. 4. Ca^{2+} oscillations in the cytosol and intracellular stores. Fluo-3 signal (dotted line, left ordinate) and Mag-fura-2 ratio (solid line, right ordinate) are represented in the graph. At time zero, the cell was under intracellular perfusion for about 8 min. Stimulation of the cell with 3 μM phenylephrine elicited Ca^{2+} oscillations and thapsigargin (1 μM) induced a release of Ca^{2+} from the IS. The effects of the two compounds are detectable in both traces.

4. Discussion

Oscillations of cytosolic free Ca^{2+} in hepatocytes is thought to involve mainly an exchange of Ca^{2+} between the IS, presumably the endoplasmic reticulum, and the cytosol. Several theoretical models describing oscillations of cytosolic free Ca^{2+} have been proposed that include the IS [4] but, because of the lack of direct measurements, the fate of Ca^{2+} in the stores remains speculative. In the present study, we describe an experimental approach enabling the simultaneous measurement of Ca^{2+} in the cytosol and IS of hepatocytes.

Fluorescent probes with different affinities for Ca^{2+} are available and generally obtainable as AM derivatives for non-disruptive cell loading. AM derivatives have the tendency to distribute not only in the cytosol but also in sub-cellular compartments of many cell types including rat hepatocytes [7]. While this property of AM molecules may lead to artifacts when pure cytosolic measurements are to be attempted, it can be viewed as a convenient way of loading probes into intracellular compartments. In the first in situ measurements of Ca^{2+} in subcellular compartments [5], cells were loaded with a low affinity Ca^{2+} probe Mag-fura-2 AM [9] originally designed as magnesium probe. After digitonin permeabilization of the plasma membrane, the cytosolic dye molecules were eliminated while the remaining fluorescent signal was associated with IS. IP_3 or thapsigargin application induced a rapid release of Ca^{2+} from the stores measured as a reduction of the Mag-fura-2 fluorescence ratio. In a recent study [6] Mag-indo-1, a dye with similar affinity for Ca^{2+} as Mag-fura-2, was used as a IS probe and, to eliminate the cytosolic dye, cells were patched in whole-cell configuration. To accelerate the diminishing of cytosolic signal Mn^{2+} was added to the pipette solution which quenched the cytosolic dye. Cytosolic Ca^{2+} variations were indirectly assessed by measuring Ca^{2+}-induced K^+ currents. In this preparation, Ca^{2+} oscillations could be seen in the IS and an associated oscillating K^+ current occurred.

Because Ca^{2+} oscillations do not occur in permeabilized cells, we chose to apply an adaptation of the patch clamp approach that would enable measurements of Ca^{2+} in the cytosol and IS simultaneously. This was achieved by loading the cells with Mag-fura-2 and patching them with a pipette containing another dye, Fluo-3. Although it is difficult to accurately calibrate the signal coming from the IS, we attempted it, using a variant of the calibration procedure originally proposed for Fura-2 [10]. The calibration experiments showed that the steady-state Ca^{2+} concentration in the IS of hepatocytes was very high, estimated at 600–700 μM, about 5,000–10,000-fold the cytosolic Ca^{2+} concentration. As a comparison, an IS free Ca^{2+} concentration of 127 μM was found in gastric gland cells [5] and 60–200 μM was estimated in gonadotropes [6]. A more accurate measurement could be achieved with a dye with higher K_d for Ca^{2+} and if the contribution of Mag-fura-2 molecules remaining in cytosol could be totally eliminated.

Hormonal stimulation by the α_1-agonist phenylephrine elicited oscillations in the cytosol and parallel transient declines in IS free Ca^{2+}. The observation that the oscillations in the two compartments happened in phase is consistent with a direct exchange between the IS and the cytosol, therefore favoring one-pool models (e.g. [3]). After spontaneous cessation of oscillations, subsequent application of the endoplasmic reticulum Ca^{2+}-pump inhibitor thapsigargin resulted in a further release of Ca^{2+} from the stores to reach a level of Ca^{2+} depletion much lower than the lowest transient decline observed during the oscillations. This indicated that during a Ca^{2+} transient, only a fraction of the total Ca^{2+} pool was released from the stores. A direct implication of this conclusion is that spike termination cannot be explained by a depletion of the stores as some models assume [11].

This approach is a powerful way to test models because it enables the definition of the minimal conditions necessary for oscillations and allows to always measure directly how Ca^{2+} flows between the IS and the cytosol. After the extensive cell dialysis obtained, most of the parameters are imposed by the pipette solution. Parameters such as buffering, threshold IP_3 concentrations can be changed, or membrane-impermeable molecules can be introduced via the patch pipette.

In conclusion, the simultaneous measurement of free Ca^{2+} in the cytosol and intracellular stores indicated that during oscillations of cytosolic Ca^{2+} reciprocal oscillations occurred in the stores. The amplitude of the periodic Ca^{2+} depletion measured in the stores compared with the extensive depletion obtained with thapsigargin indicated that only a fraction of the Ca^{2+}-pool was released during a single transient.

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