Differential control of free calcium and free zinc levels in isolated bovine liver nuclei

Stefan HECHTENBERG and Detmar BEYERSMANN*
Universität Bremen, Fachbereich Biologie/Chemie, Leobener Strasse, 2800 Bremen 33, Germany

Modulation of intracellular signal transduction by Ca^{2+} and possibly Zn^{2+} is based on an effective homoeostatic control of the corresponding free ion concentrations. We used the fluorescent indicator fura-2 to monitor concentrations of free Ca^{2+} and free Zn^{2+} in nuclei isolated from bovine liver. The nuclei showed an ATP-stimulated accumulation of intranuclear free Ca^{2+}, which was inhibited in the presence of the Ca^{2+}-pump inhibitor thapsigargin. Furthermore, uptake and intranuclear levels of free Zn^{2+} were measured after incubation with different extranuclear Zn^{2+} concentrations. There was no stimulating effect of ATP on Zn^{2+} uptake. Our data suggest that the levels of intranuclear free Ca^{2+} and free Zn^{2+} are controlled differentially. A distinct regulation of free ion levels in the nucleus may contribute to the specific control of nuclear events associated with gene transcription and cell differentiation.

INTRODUCTION

Ca^{2+} plays an important role in intracellular signal transduction. Ca^{2+} signals induced by hormones, neurotransmitters and growth factors serve as modulators of a variety of cellular functions [1,2]. For that purpose a spatial and temporal resolution of intracellular Ca^{2+} changes is critical. The intracellular distribution of free Ca^{2+} after hormone- or neurotransmitter-mediated stimulation of muscle or neuronal cells is not uniform. Studies using digital imaging microscopy of single cells with Ca^{2+}-sensitive fluorescent dyes revealed the existence of free Ca^{2+} concentration gradients between the nucleus and the cytosol in response to external stimuli [3–5]. Furthermore, nuclei isolated from rat liver have been shown to accumulate Ca^{2+} by an ATP-stimulated uptake system [6]. Moreover, sequestered Ca^{2+} can be released from nuclei in part by the intracellular messenger inositol 1,4,5-trisphosphate [7,8]. These findings suggest that the intranuclear level of free Ca^{2+} is regulated independently of the cytosolic level and participates in the specific control of Ca^{2+} dependent nuclear processes.

As for Ca^{2+}, intracellular Zn^{2+} levels seem to be under homoeostatic control. The putative regulatory role of Zn^{2+} is based on its activity in transcription factors and various DNA- and RNA-synthesizing enzymes. It has been shown that removal of Zn^{2+} from zinc finger containing transcription factors Sp1 or TF IIIa by physiological chelators such as thionein resulted in a suppression of DNA binding and transcriptional activation [9,10]. Furthermore, a decreased availability of Zn^{2+} caused an impairment of DNA synthesis associated with cell differentiation [11]. However, little is known about the distribution of free Zn^{2+} ions inside the cell and a possible control of cellular functions by free Zn^{2+}, especially in the nucleus.

The aim of the present study was to investigate mechanisms for the control of nuclear cation levels. Here, we report data on the uptake and concentration of free Ca^{2+} and free Zn^{2+} in isolated bovine liver nuclei as measured by fura-2 fluorescence and the influence of various Ca^{2+} pump inhibitors on these processes.

EXPERIMENTAL

Materials

Fura-2, fura-2 AM, ATP, EGTA, Ca^{2+}-ionophore Br-A23187 and DTPA were obtained from Sigma (Deisenhofen, Germany). Thapsigargin was from Calbiochem (Bad Soden, Germany). TPEN and tributyltin chloride were purchased from Fluka (Neu-Ulm, Germany). EDTA and NTA were obtained from Merck (Darmstadt, Germany). All other chemicals were of the highest purity commercially available.

Preparation of nuclei

Pieces of bovine liver were washed with ice-cold buffer and blotted on filter paper to remove contaminating blood. Homogenization and differential centrifugation were carried out by the method of Nicotera et al. [6]. The final pellet of highly purified nuclei was resuspended in standard incubation medium (125 mM KCl, 2 mM K_2HPO_4, 25 mM Hepes, 4 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM EDTA, 2 mM NTA, pH 7.0).

The nuclear fraction was virtually free of contamination by plasma membranes, microsomes and mitochondria, as checked by determination of marker enzyme activities (5′-nucleotidase, alkaline phosphodiesterase I, glucose-6-phosphatase, succinate–INT reductase).

Buffering of Ca^{2+} and Zn^{2+} concentrations

Free ion concentrations were adjusted in standard incubation medium. The appropriate total concentrations of Ca^{2+} or Zn^{2+} required to give the desired free ion concentrations were calculated by the computer program SPECS of Fabiato [12]. Absolute stability constants were taken from Smith and Martell [13].

Fura-2 loading of nuclei and fluorescence measurements

Isolated nuclei were preloaded with 7.5 μM fura-2 AM for 45 min at 4 °C [6]. The nuclear suspension was then washed twice and resuspended in standard incubation medium.

Abbreviations used: DTPA, diethylenetriaminepenta-acetic acid; fura-2 AM, fura-2 acetoxymethyl ester; NTA, nitrilotriacetic acid; TPEN, *NNN′N′*-tetrakis(2-pyridylmethyl)ethylenediamine.

* To whom correspondence should be addressed.

Fluorescence measurements were performed at 25 °C in a dual-wavelength fluorescence spectrometer (Perkin–Elmer LS 50). Fluorescence intensity was monitored at an emission wavelength of 510 nm, by using a pair of excitation wavelengths at 340 nm and 380 nm. Free ion concentrations were calculated from ratio data as described by Grynkiewicz et al. [14], by using the Perkin–Elmer intracellular biochemistry application software.

The apparent dissociation constants for the fura-2 complexes were determined as described by Kwan and Putney [15]. The K_d values for Ca^{2+} and Zn^{2+} in our system were calculated to be 202 nM and 1.5 nM respectively.

RESULTS AND DISCUSSION

ATP increases the Ca^{2+}-sensitive fluorescence of fura-2 in isolated bovine liver nuclei, in agreement with results obtained by Nicotera et al. [6] with rat liver nuclei. As shown in Figure 1, addition of 1 mM ATP to the nuclear suspension in the presence of 200 nM extranuclear free Ca^{2+} resulted in an accumulation of intranuclear free Ca^{2+} to a concentration of about 500 nM. The ATP-stimulated increase in intranuclear free Ca^{2+} was half-maximal at 130 ± 50 nM external free Ca^{2+}. After ATP-stimulated nuclear Ca^{2+} uptake, addition of the detergent Nonidet P-40 resulted in a total release of accumulated Ca^{2+} (Figure 1). The Ca^{2+}-ionophore Br-A23187 caused a partial decrease in intranuclear free Ca^{2+} concentration (result not shown).

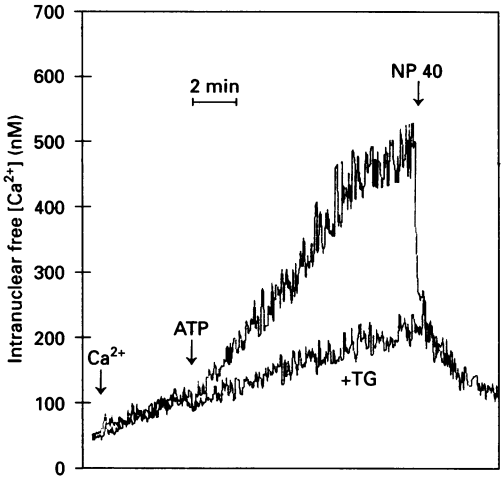

Figure 1 ATP-stimulated increase in intranuclear free Ca^{2+} concentration in the absence (upper trace) and presence of 250 nM thapsigargin (+TG; lower trace)

The following additions were made at the time intervals indicated: 200 nM free Ca^{2+}, 1 mM ATP and 0.05% Nonidet P-40 (NP 40).

Table 1 Influence of various inhibitors on ATP-stimulated accumulation of intranuclear free Ca^{2+} concentration

5 min uptake in isolated nuclei was determined in the presence of 200 nM free Ca^{2+} and various agents. Values are presented as percentages of control (mean ± S.D.).

Addition	Intranuclear Ca^{2+} accumulation (% of control)
1 mM ATP (control)	100
250 nM Thapsigargin + 1 mM ATP	30.5 ± 4.7
100 μM Vanadate + 1 mM ATP	36.1 ± 5.4
5 μM Tributyltin chloride + 1 mM ATP	44.0 ± 6.7
Passive Ca^{2+} uptake in the absence of ATP	18.4 ± 6.3

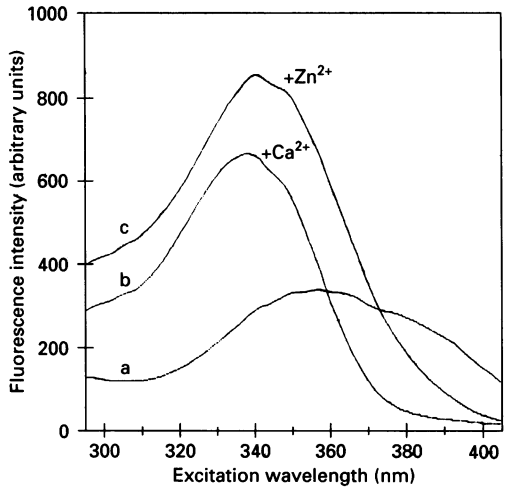

Figure 2 Fluorescence excitation spectra of fura-2 in the presence of Ca^{2+} or Zn^{2+}

Spectrum of unbound fura-2 (trace a) was obtained in standard incubation medium (125 mM KCl, 2 mM K_2HPO_4, 25 mM Hepes, 4 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM EDTA, 2.0 mM NTA, pH 7.0) containing 1 μM fura-2. Traces b and c were recorded in the presence of saturating concentrations of free Ca^{2+} (> 10 μM) and free Zn^{2+} (> 50 nM) respectively.

To characterize further the mechanism of Ca^{2+} transport into the nucleus, we investigated the influence of various agents on ATP-stimulated Ca^{2+} accumulation. Thapsigargin, a tumour-promoting sesquiterpene lactone, has been reported to inhibit ATP-driven Ca^{2+} pumps of the endo- and sarco-plasmic reticulum and to release sequestered Ca^{2+} from microsomal Ca^{2+} stores [16,17]. We now show that thapsigargin (250 nM) prevents ATP-stimulated Ca^{2+} accumulation in the nucleus nearly completely (Figure 1, lower trace). This result indicates that the nuclear Ca^{2+} transport system may have similar properties to the Ca^{2+}-uptake system of the endo-/sarco-plasmic reticulum. However, thapsigargin did not show any releasing effect on nuclei after they had been loaded with Ca^{2+} by ATP stimulation (result not shown), which confirms also the absence of contaminating microsomes in the nuclear preparation. Table 1 summarizes the results of Ca^{2+}-uptake experiments with various inhibitors. A significant decrease in Ca^{2+} accumulation was observed in the presence of the ATPase inhibitor vanadate, suggesting the involvement of ATP hydrolysis in the nuclear Ca^{2+} uptake. Furthermore, tributyltin, which has been shown to affect ion-translocating mechanisms [18], inhibited ATP-stimulated Ca^{2+} accumulation in nuclei at a concentration of 5 μM. These findings are consistent with the inhibition of sarcoplasmic-reticulum Ca^{2+}-ATPase at lower micromolar concentrations of tributyltin [18,19].

In addition to Ca^{2+}, we further investigated the nuclear uptake and level of free Zn^{2+}. Fura-2 forms complexes with other metal ions such as Ba^{2+}, Sr^{2+}, La^{3+} and Pb^{2+}, whose fluorescence properties resemble that of the Ca^{2+}–fura complex [15,20]. Zn^{2+} binding to fura-2 also produces an excitation shift similar to that of Ca^{2+}, with a pronounced increase and an accompanying decrease in fluorescence intensity at excitation wavelengths near 340 nm and 380 nm respectively (Figure 2). Therefore it is possible to determine the apparent dissociation constant for the

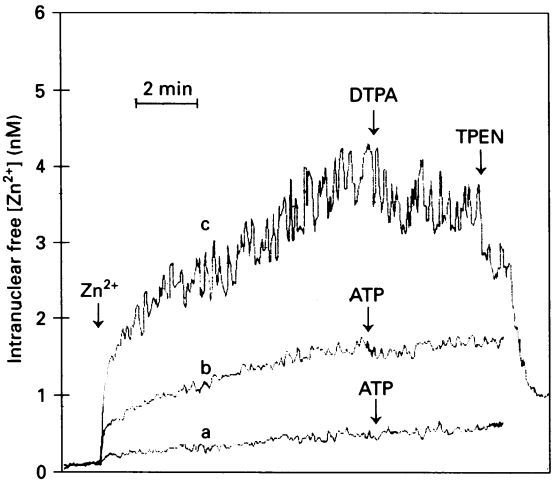

Figure 3 Increase in intranuclear free Zn^{2+} concentration and influence of ATP and different chelators

Zn^{2+} uptake in nuclei was observed in the presence of different amounts of free Zn^{2+} in the incubation medium: a, 10 nM; b, 100 nM; c, 316 nM. Further additions were made as indicated: 1 mM ATP (traces a and b); 200 μM DTPA and 200 μM TPEN (trace c). The membrane-impermeant chelator DTPA completely chelates free Zn^{2+} in the incubation medium outside the nuclei. Subsequent addition of membrane-permeant chelator TPEN results in an additional complexation of free Zn^{2+} inside the nuclei.

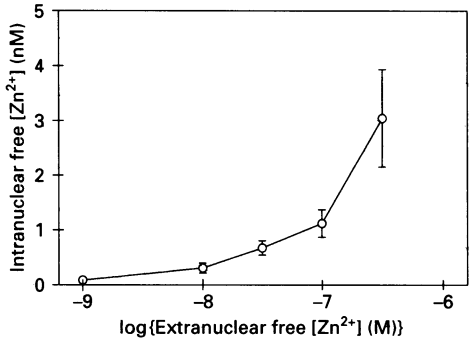

Figure 4 Intranuclear level of free Zn^{2+} after incubation for 5 min with different extranuclear free Zn^{2+} concentrations

Zn^{2+}–fura complex and to use the fura-2 method for monitoring free Zn^{2+} concentrations in isolated nuclei. Interference of Ca^{2+} with Zn^{2+}-sensitive fluorescence was ruled out, since the nuclei were depleted of Ca^{2+} by washing with chelator-containing medium. Moreover, the affinity of Zn^{2+} for the fura dye was 135 times that of Ca^{2+}.

Figure 3 (traces a–c) shows the increase in intranuclear free Zn^{2+} levels after incubation with different free Zn^{2+} concentrations in the medium. Addition of the membrane-impermeant chelator DTPA after incubation with Zn^{2+} stopped the uptake, but did not alter the acquired level of intranuclear free Zn^{2+} significantly. In contrast, subsequent addition of the membrane-permeant chelator TPEN markedly decreased the fluorescence signal (Figure 3, trace c). These results give clear evidence that the observed fura-2 signal reflects an increase in the intranuclear free Zn^{2+} concentration and excludes the possibility that the Zn^{2+}-sensitive fluorescence is produced by leakage of small amounts of fura-2 from the nuclei.

Examination of the Zn^{2+} concentrations demonstrates that the level of intranuclear free Zn^{2+} is limited compared with the extranuclear concentration of free Zn^{2+} offered in the incubation medium (Figure 4). This is confirmed by the observation that solubilization of the nuclear membrane by addition of the detergent Nonidet P-40 produced an increase in the fluorescence signal (results not shown).

In contrast with the results obtained with Ca^{2+}, no stimulation of Zn^{2+} uptake was observed by addition of ATP (Figure 3, traces a and b). Furthermore, the increase in intranuclear free Zn^{2+} concentration was not altered in the presence of 250 nM thapsigargin (results not shown).

Hence it is obvious that the intranuclear levels of free Ca^{2+} and free Zn^{2+} are controlled differentially. Ca^{2+} is accumulated by an ATP-stimulated thapsigargin-sensitive transport system, which provides a mechanism for the distinct regulation of the intranuclear free Ca^{2+} concentration in co-operation with other elements of a putative nuclear signal-transduction machinery. The control of this level is a basic requirement for the modulation of Ca^{2+}-regulated processes in the nucleus, such as activation of Ca^{2+}-dependent endonuclease [21] or nuclear protein kinase [22]. The relative distribution of free Ca^{2+} between cytoplasm and nucleoplasm is different in various mammalian systems. Whereas nuclear Ca^{2+} concentration is higher than cytoplasmic Ca^{2+} in smooth-muscle cells [3], neuronal cells [4] and erythropoietin-stimulated erythroblasts [5], the nuclear Ca^{2+} concentration is lower than cytoplasmic Ca^{2+} in cultured hepatocytes [23]. In the latter system, stimulation by hormones resulted in an increase in both nuclear and cytoplasmic Ca^{2+}, and the nuclear-to-cytoplasmic Ca^{2+} gradient was preserved. The reasons for the different distribution of Ca^{2+} in various cell types have not yet been elucidated.

Whereas there exist several lines of evidence for a regulatory role of nuclear free Ca^{2+}, a regulatory function of free Zn^{2+} in cell nuclei is still speculative. Our data show that the level of free Zn^{2+} in the nucleus is limited, suggesting a barrier function of the nuclear membrane and/or large buffer capacities of intranuclear Zn^{2+}-binding sites. The observed nuclear uptake of Zn^{2+} may be mediated by cation-selective channels in the nuclear membrane, which have been reported for other ions [24,25]. Since reliable data on intracellular free Zn^{2+} concentrations *in vivo* are not yet available, it is difficult to assign the observed intranuclear levels to distinct Zn^{2+}-regulated functions. In particular, a putative stimulation of Zn^{2+} release in nuclei has still to be demonstrated. Nevertheless, a spatial resolution and control of intracellular free-Zn^{2+} homoeostasis, especially in the nucleus, is a substantial prerequisite for the postulated role of Zn^{2+} as a regulator of cell differentiation and growth.

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