Interference of Cadmium with ATP-stimulated Nuclear Calcium Uptake

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The spatial and temporal regulation of intracellular free Ca\textsuperscript{2+} serves as a modulator of signal transduction pathways involved in cell growth and differentiation. Thus, interference of metals with intracellular Ca\textsuperscript{2+} homeostasis has been considered as a target of toxic action. We used the fluorescence indicator fura-2 to monitor the level of free Ca\textsuperscript{2+} in isolated bovine liver nuclei. Nuclei accumulated Ca\textsuperscript{2+} by an ATP-stimulated Ca\textsuperscript{2+} uptake system, which is sensitive to inhibition by thapsigargin, a specific inhibitor of intracellular Ca\textsuperscript{2+}-ATPases. Preincubation of nuclei with nanomolar concentrations of free Cd\textsuperscript{2+} resulted in a dose-dependent inhibition of ATP-dependent nuclear Ca\textsuperscript{2+} uptake. We conclude that impairment of nuclear Ca\textsuperscript{2+} regulation caused by Cd\textsuperscript{2+} provokes alterations in nuclear events related to gene expression and cell proliferation. — Environ Health Perspect 102(Suppl 3):265–267 (1994).

Key words: liver nuclei, Ca\textsuperscript{2+} transport, Ca\textsuperscript{2+}-ATPase, thapsigargin, cadmium

Introduction

Beside direct genotoxic actions, epigenetic mechanisms have gained considerable attention for the interpretation of carcinogenic action of metals. Therefore, the interaction of metal ions with cellular signaling is of particular interest (1–3). Transient changes in intracellular free Ca\textsuperscript{2+} concentration induced by hormones, neurotransmitters, or growth factors modulate a variety of physiologic processes such as cell proliferation and differentiation. Thereby, it seems obvious that the toxic action of metal ions may be mediated by interference with Ca\textsuperscript{2+}-modulated signal transduction pathways.

Cd\textsuperscript{2+} has been shown to inhibit Ca\textsuperscript{2+} transport at several cellular sites with different consequences. Cd\textsuperscript{2+} blocks Ca\textsuperscript{2+} influx via the plasma membrane by displacing Ca\textsuperscript{2+} from its transport site in Ca\textsuperscript{2+} channels (4) or by interaction with the receptor-operated Ca\textsuperscript{2+} influx system (5). However, Cd\textsuperscript{2+} inhibits Ca\textsuperscript{2+} extrusion from cells mediated by the plasma membrane Ca\textsuperscript{2+} pump (6,7), resulting in a disturbed intracellular Ca\textsuperscript{2+} homeostasis and increasing cytosolic free Ca\textsuperscript{2+} levels. The same consequences could be evoked by interaction of Cd\textsuperscript{2+} with Ca\textsuperscript{2+} transport into intracellular stores, such as interference with hepatic micromosal Ca\textsuperscript{2+} sequestration (8) or inhibition of sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (9).

Several lines of evidence suggest that the level of free Ca\textsuperscript{2+} in the nucleus is important for the regulation of nuclear functions such as DNA replication, gene transcription, phosphorylation and the dephosphorylation of nuclear proteins, and DNA fragmentation (10). Recently, two systems for the regulation of nuclear free Ca\textsuperscript{2+} have been described in isolated rat liver nuclei: Ca\textsuperscript{2+} can be accumulated by an ATP-stimulated uptake system (11) and released in an IP\textsubscript{3}-induced manner (12,13). The ATP-dependent Ca\textsuperscript{2+} uptake is mediated by a Ca\textsuperscript{2+} transporting pump closely related to the endoplasmic/sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (14).

The present study was undertaken to evaluate the effect of Cd\textsuperscript{2+} on the ATP-stimulated Ca\textsuperscript{2+} transport system of isolated bovine liver nuclei.

Materials and Methods

Fura-2 acetoxyethyl ester, Ca\textsuperscript{2+}-ionophore A 23187, Nonidet P-40, and Verapamil were obtained from Sigma (Deisenhofen, Germany). Thapsigargin was purchased from Calbiochem (Bad Soden, Germany), tributyltin chloride from Fluka (Neu-Ulm, Germany). All other reagents were of highest analytical 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. (11). The final pellet of highly purified nuclei was resuspended in standard incubation medium (125 mM KCl, 2 mM K\textsubscript{2}HPO\textsubscript{4}, 25 mM Hepes, 4 mM MgCl\textsubscript{2}, 0.5 mM EGTA, 0.5 mM EDTA, 2 mM NTA, pH 7.0).

The contamination of isolated nuclei by plasma membranes, microsomes, and mitochondria was checked by the determination of marker enzyme activities. The total activities of 5'-nucleotidase, alkaline phosphodiesterase 1, glucose-6-phosphatase, and succinate-INT-reductase found in the nuclear fraction were less than 0.5% of those found in the initial homogenate.

Buffering of Ca\textsuperscript{2+} and Cd\textsuperscript{2+} Concentrations

Free ion concentrations were adjusted in standard incubation medium. The appropriate total concentrations of Ca\textsuperscript{2+} or Cd\textsuperscript{2+} required to give the desired free ion concentrations were calculated by the SPECS computer program of Fabiato (15). Absolute stability constants were taken from Smith and Martell (16).

Fura-2 Loading of Nuclei and Fluorescence Measurements of Intranuclear Free Ca\textsuperscript{2+} Concentration

Loading of isolated nuclei with fura-2 acetoxyethyl ester and fluorescence measurements were carried out as described previously (17).

Preincubation of Nuclei with Cd\textsuperscript{2+}

Fura-2-loaded nuclei were resuspended in standard incubation medium suppli-
mplemented with the desired concentration of Cd²⁺ and incubated for 10 min. Nuclei were then pelleted by centrifugation at 700g for 3 min. Nuclear pellets were washed twice with chelator-containing medium before starting the fluorescence measurement.

**Determination of Intracellular Total Cd by Atomic Absorption Spectroscopy**

Isolated nuclei, but not loaded with fura-2, were treated with Cd²⁺ as described in the preincubation procedure. Nuclear pellets were then mineralized by addition of 200 μl freshly prepared acid solution (65% [HNO₃] + 30% [H₂O₂], 1:1) and taken to dryness overnight at 85°C. The residues were redissolved in 0.2% HNO₃ and analyzed for cadmium with a Perkin Elmer 2380 atomic absorption spectrophotometer equipped with a HGA 400 graphite furnace (Überlingen, Germany).

**Results**

Isolated bovine liver nuclei loaded with fura-2 showed a marked increase in Ca²⁺-sensitive fluorescence by addition of 1 mM ATP. Whereas passive Ca²⁺ uptake in the presence of 200 nM extranuclear free Ca²⁺ resulted in an intranuclear concentration of only about 100 nM after 5 min, ATP caused an increase of intranuclear free Ca²⁺ to a level of about 400 nM. Accumulated free Ca²⁺ was releasable by addition of Ca²⁺-ionophor Br-A23187 (Figure 1).

Figure 2 summarizes the results of Ca²⁺ uptake experiments in the presence of various inhibitors. Thapsigargin, a tumor promoting sesquiterpene lactone known to inhibit ATP-driven Ca²⁺ pumps of the endo- and sarcoplasmic reticulum (18,19), prevented ATP-stimulated accumulation of free Ca²⁺ in isolated nuclei completely. Furthermore, significant decrease of ATP-stimulated nuclear Ca²⁺ uptake was observed in the presence of the ATPase inhibitors vanadate and tributyltin. In contrast, verapamil, an inhibitor of Ca²⁺ transport across the plasma membrane through Ca²⁺ channels (20,21), did not produce a significant decrease in ATP-stimulated nuclear Ca²⁺ accumulation.

Since Cd²⁺ interferes with Ca²⁺-sensitive fluorescence properties of fura-2, it was not possible to determine the influence of Cd²⁺ on ATP-stimulated Ca²⁺ uptake in fura-2-loaded nuclei directly. Therefore, it was necessary to preincubate the nuclear suspension with various concentrations of free Cd²⁺ and to wash the nuclei with chelator-containing medium before starting the Ca²⁺ sensitive fluorescence measurement. Figure 3 shows that preincubation of the nuclear suspension with Cd²⁺ decreased the ATP-stimulated Ca²⁺ uptake. The inhibitory effect of Cd²⁺ depends on the concentration of free metal ion employed during the preincubation procedure (Figure 4). Half-maximal inhibition of nuclear Ca²⁺ accumulation is reached at about 10 nM free Cd²⁺.

Additionally, we studied the uptake of Cd²⁺ in isolated nuclei in the concentration range, where Ca²⁺ uptake is inhibited.
The data in Figure 5, obtained from determinations by atomic absorption spectroscopy, demonstrate that significant amounts of intranuclear bound Cd were detectable from about 1 pM free Cd\(^{2+}\) in the incubation medium. The level of total Cd in nuclei is elevated with increasing concentrations of extranuclear free Cd\(^{2+}\).

**Discussion**

In agreement with previous reports (11,17), incubation of isolated liver nuclei with ATP led to an accumulation of intranuclear free Ca\(^{2+}\) concentration. The ATP-stimulated Ca\(^{2+}\) uptake system is sensitive to the inhibition by thapsigargin, a specific inhibitor of intracellular Ca\(^{2+}\) ATPases. This result confirms recent findings that the nuclear membrane contains a Ca\(^{2+}\)-transporting pump that is closely related to that of the endoplasmic reticulum (14). The active Ca\(^{2+}\) transport system provides a mechanism for the regulation of intranuclear free Ca\(^{2+}\) and may be critical for the control of Ca\(^{2+}\) modulated nuclear functions.

The results obtained after preincubation of nuclear suspension with Cd\(^{2+}\) show that ATP-stimulated nuclear Ca\(^{2+}\) uptake is sensitive to inhibition by the heavy metal ion. Significant inhibition is already observed at low nanomolar concentrations of free Cd\(^{2+}\). The inhibitory range of free Cd\(^{2+}\) is comparable to that reported for the inhibition of plasma membrane Ca\(^{2+}\) ATPase (6,7). Since Cd\(^{2+}\) has an ionic radius similar to Ca\(^{2+}\), it is able to replace Ca\(^{2+}\) from its intracellular transport and binding sites. Therefore, the inhibitory action of Cd\(^{2+}\) on ATP-stimulated nuclear Ca\(^{2+}\) transport may be a result of blocking the transport binding site of the nuclear Ca\(^{2+}\) pump. Furthermore, the data from determination of total Cd demonstrate that the toxic metal ion is able to enter the nucleus itself, depending on the concentration of free Cd\(^{2+}\) adjusted in the incubation medium. As a consequence, Cd\(^{2+}\) occupies binding sites of intranuclear Ca\(^{2+}\) stores, so that the capacity of the nucleus to sequester Ca\(^{2+}\) is reduced.

Alterations in nuclear Ca\(^{2+}\) homeostasis caused by Cd\(^{2+}\) would disturb the Ca\(^{2+}\)-dependent regulation of DNA-replication and gene transcription. It is known that Ca\(^{2+}\) controls the expression of growth- and transformation-related genes, such as c-fos and c-jun (22). Therefore, the impairment of nuclear Ca\(^{2+}\) regulation by Cd\(^{2+}\) may have consequences for the regulation of cell proliferation and tumorigenesis.

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