Calcium transported to isolated rat liver nuclei by nicotinamide adenine dinucleotide is insensitive to thapsigargin

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Abstract Calcium uptake by isolated nuclei was mediated by nicotinamide adenine dinucleotide. Oxidized nicotinamide nucleotide analogues were more effective mediators of nuclear calcium uptake. Thapsigargin inhibited ATP-mediated nuclear calcium transport without affecting NAD-mediated nuclear calcium uptake. Whilst DBHQ did not influence ATP-induced calcium transport, it did stimulate NAD-mediated nuclear calcium entry. Calcium channel blockers did not influence the action of NAD. This study provides a further mechanism for nuclear calcium transport regulated by changes in the cytosolic NAD^+/NADH ratio.

Key words: Nuclear calcium; Ca^{2+}-ATPase; Thapsigargin; NAD

1. Introduction

The regulation of intracellular calcium has been extensively studied [1,2]. At the present moment the relationship between cytosolic calcium changes and nuclear calcium levels [2-6] has drawn considerable attention. Calcium is implicated in diverse nuclear functions including regulation of transcription factors [7], DNA repair [8], mitosis and meiosis [9,10]. Passive calcium diffusion to the nucleus [3] is contested in view of calcium regulated transport mechanisms involving ATP [11] and inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) [12]. Extranuclear calcium concentrations determine the mode of nuclear calcium entry [13]. The nuclear envelope seems to serve as a nucleocytoplasmic barrier to free diffusion of calcium when external free calcium concentrations exceed 300 nM [14]. Nuclei contain their own phosphatidylinositol metabolic enzymes [15]. Changes in levels of nuclear diacylglycerol (DAG) during differentiation have been proposed [16]. Inositol 1,4,5-trisphosphate receptor (IP_3R) initially located to the intact nucleus [17] has now been shown to be associated with the inner nuclear membrane [13,18]. IP_3R can be phosphorylated by nuclear protein kinase C [19]. The role of $InsP_3$ in calcium movement within the nucleus is well founded [20].

Data presented in this report reveal that NAD was able to mediate nuclear calcium uptake. The amount of calcium brought into the nucleus by NAD was comparable with the calcium transported to the nucleus by ATP. In the study reported here, thapsigargin, a specific inhibitor of endoplasmic reticulum (ER) Ca^{2+}-ATPase [21,22], inhibited the ATP-mediated nuclear calcium uptake without effecting calcium uptake mediated by NAD. DBHQ (2,5-di(*t*-butyl)-1,4-benzohydroquinone), another class of Ca^{2+}-ATPase inhibitor [23], did not influence ATP-mediated calcium transport to the nuclei, but enhanced the effect of NAD on nuclear calcium entry. The study reported here provides a further mechanism of nuclear calcium transport triggered by NAD and which may be regulated through changes in the cytosolic NAD^+/NADH ratio.

2. Materials and methods

Reagents: DBHQ was purchased from Aldrich, thapsigargin, heparin, verapamil, *N*-ethylmaleimide (NEM), diltiazem, DMSO (dimethyl sulfoxide), NAD and analogues were from Sigma, France.

2.1. Isolation of rat liver nuclei

Rat liver nuclei were isolated as described earlier [24]. Briefly, small pieces of freshly removed rat liver were homogenized in 6–8 vols. of a medium containing 1.3 M sucrose, 1.0 mM $MgCl_2$ and 10 mM potassium phosphate, pH 6.8. After filtering through four layers of cheesecloth the homogenate was centrifuged for 15 min at $1000\times g$. The pellet was resuspended in the same medium and mixed with a 2.4 M sucrose medium (2.4 M sucrose, 1.0 mM $MgCl_2$ and 10 mM potassium phosphate, pH 6.8), so as to give a final sucrose concentration of 2.2 M and centrifuged at $100\,000\times g$ for 1 h. The resulting nuclear pellet was resuspended in a 0.25 M sucrose medium (0.25 M sucrose, 4.0 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5), and centrifuged for 10 min at $1000\times g$. The pellet constituted the final nuclear preparation and was devoid of any microsomal, mitochondrial or plasma membrane contaminants as documented earlier [13,17,25].

2.2. Calcium ($^{45}Ca^{2+}$) uptake and release

Isolated nuclei were suspended in a medium containing 0.25 M sucrose, 2 mM EGTA, 2 mM EDTA, 4 mM K_2HPO_4, 4 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5, and 2.08 mM $CaCl_2$ in the absence of ATP or 2.14 mM $CaCl_2$ in the presence of ATP. The free calcium concentration calculated as in [26] was 1 μM (unless otherwise indicated) and $^{45}Ca^{2+}$ was 2 μCi/ml. $^{45}Ca^{2+}$ uptake was determined for 5 min (unless otherwise indicated) at 37°C, in a final volume of 300 μl. Reactions were terminated by filtration under vacuum over GF/B (Whatman) glass filters followed by a rapid rinsing with 5 ml of ice-cold uptake medium (as above) devoid of $CaCl_2$. The filters were placed in 5 ml of Biofluor liquid scintillator and the amount of $^{45}Ca^{2+}$ trapped was determined. For the release experiments, $^{45}Ca^{2+}$ loaded nuclei were placed on ice (0°C). To this 20 mM glucose and 10 units/ml of hexokinase (ATP dissipating system) were added and incubated for 2 min at 37°C. $InsP_3$ was added to the nuclei for the indicated times and nuclear material was filtered as described above. $^{45}Ca^{2+}$ release, after a given time was defined as the amount of calcium at time zero minus the radioactivity trapped on the filter paper after the addition of $InsP_3$, at indicated time.

3. Results

3.1. $^{45}Ca^{2+}$ uptake by isolated nuclei

Fig. 1 shows calcium uptake by isolated nuclei mediated by ATP or NAD to a comparable extent. NAD-mediated nuclear Ca^{2+} uptake was not inhibited by thapsigargin, but enhanced by DBHQ (Fig. 2). ATP-mediated nuclear calcium transport

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was inhibited by thapsigargin (Fig. 2A) and was insensitive to DBHQ (Fig. 2B).

Fig. 3A depicts the time-dependent nuclear calcium entry mediated by NAD. The calcium uptake was maximum at 5 min. The increase in external free calcium concentrations from 1.5 up to 10 μM (Fig. 3B) did not change the extent of NAD-mediated calcium uptake. ATP-mediated calcium transport was dependent on the concentrations of free calcium bathing the isolated nuclei (Fig. 3B).

Oxidized (NAD, NADP) and reduced (NADH, NADPH) nicotinamide analogues were examined for mediating nuclear calcium uptake (Table 1). Data revealed that the oxidized forms of the nicotinamide analogues were more effective in nuclear calcium uptake. When calcium uptake was evaluated in terms of oxidized/reduced analogues the ratio of 1.5 was found with either NAD^+/NADH or $NADP^+$/NADPH.

Verapamil [27], *N*-ethylmaleimide [26] and diltiazem [29] were tested for their influence on nuclear calcium uptake (Table 2). The profile of inhibitory effect appeared similar for each of the inhibitors tested. However, none of these compounds effectively inhibited NAD-mediated nuclear calcium entry. Heparin did not effect NAD-mediated calcium uptake (data not shown).

The nuclei loaded with $^{45}Ca^{2+}$ in the presence of NAD were further incubated (Fig. 4) with digitonin (0.5 μM) or sodium citrate (1%) for various times. Digitonin has been used as a permeabilizing agent [27] and sodium citrate has been shown to selectively disrupt the outer nuclear membrane [13,31,32]. Neither of these agents changed the actual levels of nuclear calcium, suggesting that calcium was still trapped within the nucleus structure (discussed further below).

3.2. Nuclear calcium release

When calcium loaded nuclei were preincubated with an ATP dissipating system (glucose+hexokinase) calcium release (Fig. 5) was seen with $InsP_3$ within 30 s. The action of $InsP_3$ was instantaneous and sensitive to heparin. Thapsigargin did not affect $InsP_3$-induced calcium release. When thapsigargin was added to the calcium loaded nuclei instead of $InsP_3$ no calcium release was seen.

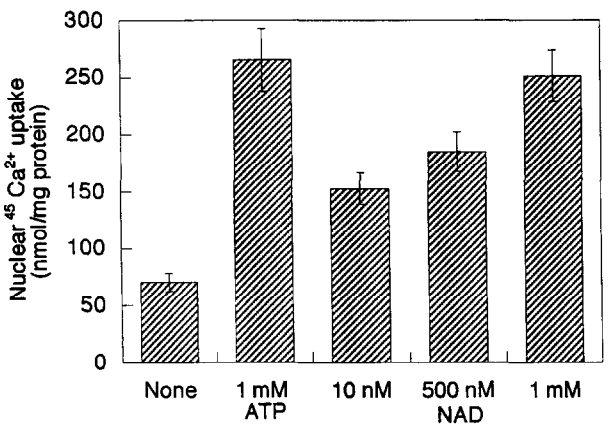


Fig. 1. NAD-mediated nuclear calcium uptake. Isolated nuclei were incubated with NAD or ATP in calcium uptake buffer as described under section 2. The external free calcium concentration in the incubation medium was maintained at 1 μM with traces of $^{45}Ca^{2+}$. Calcium uptake was terminated by rapid filtration under vacuum over GF/B (Whatman) glass fiber filters followed by rapid rinsing with 5 ml of ice-cold medium. Filters were transferred to 5 ml biofluor liquid scintillation vials, and $^{45}Ca^{2+}$ trapped on the filters was determined by spectrometry. Each experiment was performed in triplicate. Total number of experiments was 5. None corresponds to value without NAD or ATP.

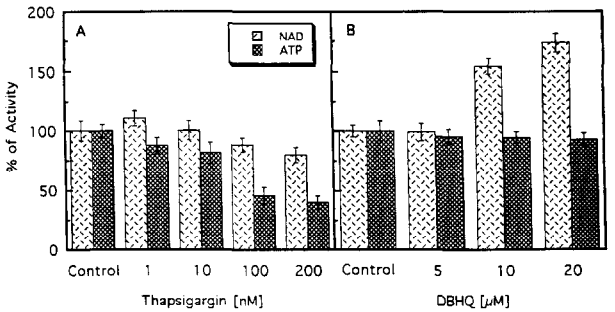


Fig. 2. Effect of thapsigargin (A) and DBHQ (B) on nuclear calcium uptake. The experimental conditions were the same as those described in the legend to Fig. 1. Thapsigargin or DBHQ was added together with the mediator (NAD or ATP). The control represents calcium uptake without inhibitors. The 100% values of uptake correspond to 265.34 ± 27.50 and 251.18 ± 22.70 nmol/mg protein for ATP and NAD, respectively. Results represent the mean of four independent experiments each carried out in triplicates.

4. Discussion

The mechanism by which calcium is transported to the nucleus is currently a subject of debate [3-5,13,18,33]. Evidence has been accumulating demonstrating that calcium movement, in and out of the nucleus, is a regulated process. It is agreed that ATP [11,17,20] and $InsP_4$ [12,34] mediate external calcium entry into the nucleus, presumably into the nuclear envelope. The $InsP_3$ releases calcium from the nuclear envelope to the nuclear matrix [13,19,20]. In the study reported here we document that NAD (also other analogues) was able to induce calcium entry to the nuclei. The action of NAD was time dependent and 5 min was found to be optimum for maximum calcium uptake (Fig. 3A). The amount of calcium brought into the nucleus by NAD was of a level comparable to that mediated by ATP (Fig. 1). Nuclear calcium uptake mediated by NAD increased up to 1 μM free calcium. Any further rise in external free calcium level did not affect the amount of calcium transported by NAD (Fig. 3B), whereas calcium levels above 1 μM, i.e. 5 or 10 μM impaired the action of ATP.

Thapsigargin, a tumor promoter and a specific inhibitor of SERCA-ATPase [21,22], inhibited ATP-mediated nuclear calcium transport without influencing the nuclear calcium entry mediated by NAD (Fig. 2A). DBHQ has been proposed [23] to inhibit selectively microsomal Ca^{2+}-ATPase. We document

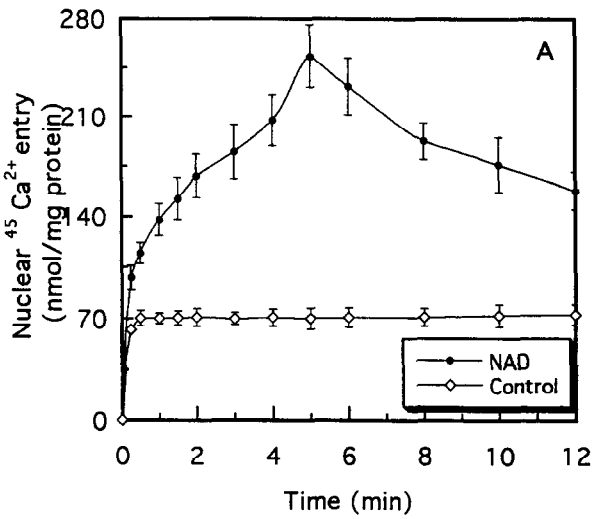
Table 1
Nicotinamide analogue-mediated nuclear calcium uptake

Additions	$^{45}Ca^{2+}$ uptake (nmol/mg protein)	Oxidized/reduced analogues (ratio of effect)
None	70.24 ± 8.30	
NAD	251.20 ± 23.00	1.49 ± 0.15
NADH	167.60 ± 18.70	
NADP	223.60 ± 16.20	1.50 ± 0.17
NADPH	148.90 ± 15.30	

The conditions for nuclear calcium uptake were the same as those described in the legend to Fig. 1. Concentration of NAD and analogues used was 1 mM. These data are based on three independent experiments, each performed in triplicates with indicated S.E.M. values.

here that DBHQ did not inhibit ATP-induced nuclear calcium uptake, but stimulated the action of NAD in nuclear calcium entry (Fig. 2B). These data support earlier reports that the endoplasmic reticulum and nuclear Ca^{2+}-ATPase are structurally identical [35] but functionally dissimilar [36]. $InsP_3$-mediated nuclear calcium release (Fig. 5) was found to be insensitive to thapsigargin.

Another interesting aspect that emerged from this study concerns the oxidized nicotinamide analogues (NAD or NADP) being more effective mediators of calcium entry into the nucleus as compared with NADH or NADPH (Table 1). This tempts up to postulate that cytosolic NAD^+/NADH levels may trigger nuclear calcium entry under in situ conditions which may provide a link between the metabolic status of a cell and nuclear calcium content. These results are based on studies conducted with isolated nuclei. The techniques for calcium studies under in vivo conditions are currently being developed [37]. The action of NAD on nuclear calcium uptake has not been demonstrated before, although it was shown that oxidation of pyridine nucleotides caused mitochondrial calcium release [38] and enhanced $InsP_3$-mediated cytosolic calcium wave activity [39].



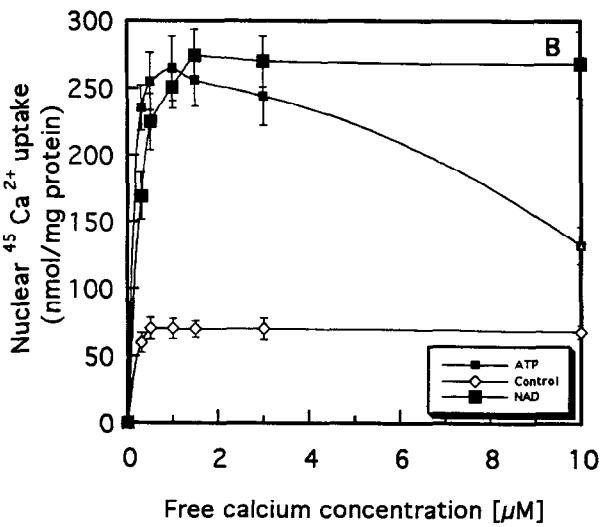


Fig. 3. NAD-mediated nuclear calcium entry as a function of time (A). Effect of external free calcium concentration on nuclear calcium uptake (B). The conditions for calcium uptake were the same as those described in the legend to Fig. 1. The amount of calcium was added according to the calculation [26] so as to give the free calcium concentration as indicated (B). Data represent the mean of four independent experiments each performed in triplicate.

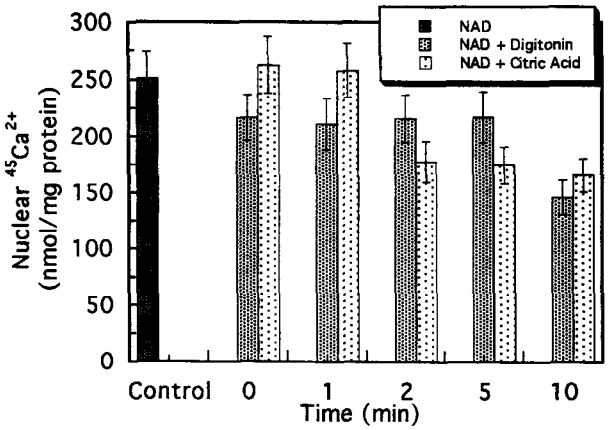


Fig. 4. Effect of digitonin and sodium citrate on nuclei loaded with $^{45}Ca^{2+}$ by NAD. Nuclei were loaded with $^{45}Ca^{2+}$ in the presence of 1 mM NAD for 5 min at 37°C. Digitonin (0.5 μM final) or sodium citrate (1% final) was added and the reaction was terminated at the indicated time. The rest of the procedure was the same as described in the legend to Fig. 1. Control represents nuclear calcium level obtained with NAD at 5 min.

A number of calcium channel blockers examined did not inhibit NAD-mediated nuclear calcium uptake (Table 2). This led us to believe that the action of NAD does not involve calcium channels, if indeed such channels are located on the nuclear membrane.

When NAD-mediated $^{45}Ca^{2+}$ loaded nuclei were treated with digitonin (a permeabilizing agent) or sodium citrate (an agent having a selective action on the outer nuclear membrane) for various times, almost no loss of $^{45}Ca^{2+}$ was seen (Fig. 4). This implies that calcium brought into the nucleus by NAD was not accumulated in the nuclear envelope.

These observations suggest that NAD mediates nuclear calcium uptake directly into the nuclear matrix. In this way, the action of NAD in nuclear calcium movement is unique and distinct from that of ATP or $InsP_4$. It may be recalled here

Table 2
Effect of inhibitors on NAD-mediated nuclear calcium uptake

Additions	$^{45}Ca^{2+}$ uptake (nmol/mg protein)
None	70.24 ± 8.30
NAD	251.20 ± 23.00
NAD+verapamil 10 μM	268.40 ± 24.00
NAD+verapamil 100 μM	187.10 ± 16.30
NAD+NEM 10 μM	252.20 ± 21.50
NAD+NEM 100 μM	167.20 ± 18.40
NAD+diltiazem 10 μM	172.50 ± 16.40
NAD+diltiazem 100 μM	196.50 ± 21.00

The experimental conditions were the same as those described in the legend to Fig. 1. Results represent the mean of three independent experiments performed in triplicates with indicated S.E.M. values.

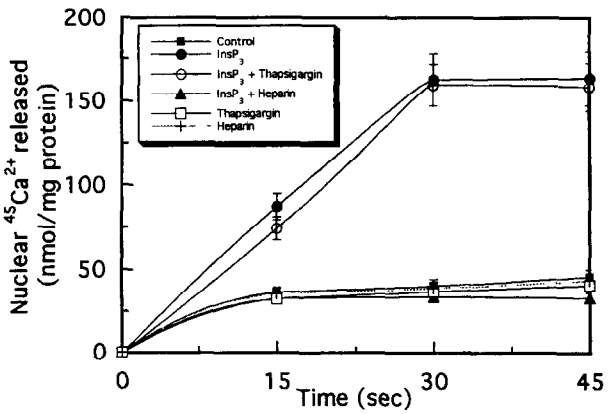


Fig. 5. Effect of thapsigargin or heparin on $InsP_3$-mediated nuclear calcium release. Nuclei were loaded with $^{45}Ca^{2+}$ in the presence of ATP as described under section 2. Calcium loading was terminated by chilling the tubes on ice. 20 mM glucose and 10 units/ml hexokinase were added to dissipate any excess ATP [12]. The release of $^{45}Ca^{2+}$ was initiated by the molecules as indicated followed by rapid filtration and rinsing as described in the legend to Fig. 1. $^{45}Ca^{2+}$ release was defined as the radioactivity loaded (time 0) minus the radioactivity trapped on the filter paper. This experiment was repeated four times with each value derived from quadruplicates.

that it is generally agreed that ATP or $InsP_4$ triggers calcium entry into the nuclear envelope.

In conclusion, we record here that NAD mediated nuclear calcium uptake, presumably to the nuclear matrix, was insensitive to thapsigargin and was stimulated by DBHQ. External free calcium levels beyond 1 μM did not influence the action of NAD. The proposed action of NAD as a mediator of nuclear calcium uptake levels provides yet another dimension towards the understanding of a possible link between various cellular compartments implicated in calcium waves.

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