Serine base-exchange in rat liver nuclei

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Received 25 July 2000; revised 7 September 2000; accepted 7 September 2000

Edited by Shozo Yamamoto

Abstract It has been shown that the incorporation of [^{14}C]serine into phosphatidylserine (PS) in isolated rat liver nuclei is intrinsic to this organelle as attested by marker enzyme activity. Serine incorporation into PS was the highest in nuclei depleted of the outer membrane of the nuclear envelope (nucleoplasts) and negligible in the outer membrane. Trypsin treatment of nucleoplasts caused a strong inactivation of PS synthesis and only a moderate one of the NAD pyrophosphorylase activity, the marker enzyme of the inner nuclear membrane. We suggest that the serine base-exchange enzyme is located in the inner membrane of the nuclear envelope and accessible from the periplasmic surface of this membrane.

Key words: Serine base-exchange; Phosphatidylserine; Nucleus; Rat liver

1. Introduction

There is now compelling evidence that the nuclear lipid metabolism participates in signalling cascades [1–3]. It is also well established that calcium signals exist in the cell nucleus. The source of nuclear calcium signals has been proposed to be the nuclear envelope [4,5]. It has also been proposed that nuclei have an intranuclear inositol lipid metabolism mechanism equivalent to those found in the cytosol and the plasma membrane [2,3]. Almost all isoforms of protein kinase C (PKC) have been identified in nuclei in a variety of mammalian cell systems [6]. For their full activity all of them require phosphatidylserine (PS) [7].

In mammalian cells, PS is synthesised by the base-exchange reaction in which serine is directly exchanged for the amino alcohol moiety of pre-existing phospholipids, particularly phosphatidylcholine and phosphatidylethanolamine [8]. The reaction is independent of metabolic energy, characterised by a requirement for a relatively high (mM) concentration of Ca^{2+} [9] and occurs mainly in the endoplasmic reticulum (ER) [9,10]. We have previously found that Ca^{2+} liberation from the ER Ca^{2+} stores strongly inhibits this reaction [11–14]. Although in mammalian nuclei PS represents about 9% of nuclear lipids [15], PS synthesis in this organelle has been somewhat ignored [1]. Van Golde et al. [16] and Jelsema and Morré [17] reported the presence of the serine base-exchange activity in nuclei, but a more detailed study on the subnuclear enzyme localisation has not been performed to date.

Therefore, the present study was undertaken to elucidate the nuclear compartmentalisation of the serine base-exchange reaction. The results demonstrate, what is to our knowledge, the first evidence that the Ca^{2+}-dependent PS synthesis is mainly localised in the inner membrane of the nuclear envelope.

2. Materials and methods

2.1. Materials

4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), sucrose, trichloroacetic acid (TCA), trypsin (TPCK-treated) from bovine pancreas and soybean trypsin inhibitor (type II), PS from bovine brain and Fiske-Subarrow reducer were from Sigma (St. Louis, MO, USA). Glucose-6-phosphate (G-6-P) was obtained from Serva (Heidelberg, Germany). L-[U-^{14}C]serine (specific activity 151 mCi/mmol) was obtained from Amersham International (Buckinghamshire, UK). Ninhydrin was purchased from Calbiochem (La Jolla, CA, USA). Silica gel thin layer chromatography (TLC) aluminium plates were from Merck (Darmstadt, Germany). All other reagents were of analytical grade.

2.2. Isolation of nuclei and subnuclear fractions

Intact nuclei from rat liver were isolated as described by Vann et al. [18]. Livers were homogenised in cold buffer A (10 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 25 mM KCl). The filtered homogenate was mixed with buffer B (10 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 2.4 M sucrose) to obtain 250 mM final sucrose concentration and used further for enzyme assays. Part of the homogenate was diluted with buffer C (10 mM HEPES, pH 7.5, 2 mM $MgCl_2$, 250 mM sucrose) and centrifuged for 15 min at $10\,000\times g$. The supernatant was further centrifuged for 1 h at $100\,000\times g$ to sediment microsomes. The rest of the homogenate was mixed with buffer D (10 mM HEPES, pH 7.5, 2 mM $MgCl_2$, 2.3 M sucrose) to obtain a final sucrose concentration of 1.62 M, overlaid on 7.5 ml of buffer D and centrifuged for 30 min at $100\,000\times g$. The pelleted nuclei were washed by sedimenting twice in buffer D at $165\times g$ for 5 min. The resulting intact nuclei were treated with 1% (w/v) sodium citrate for 30 min on ice to prepare nuclei devoid of the outer nuclear membranes (nucleoplasts) as described by Humbert et al. [19]. Nucleoplasts were sedimented at $500\times g$ for 15 min and washed once in buffer C. Crude outer nuclear membranes were sedimented at $100\,000\times g$ for 30 min. Protein was determined by a modification [20] of the procedure of Lowry et al. [21].

2.3. Serine base-exchange activity

The assay of the serine base-exchange reaction was performed according to the method used for rat liver microsomes [22] with modification. The incubation medium contained 0.5 mg protein of the indicated fraction, 100 mM sucrose, 50 mM HEPES, pH 7.4, 1 mM $CaCl_2$, 0.8 mM $MgCl_2$ and 50 μM L-[U-^{14}C]serine (specific activity 9 mCi/mmol), in a total volume of 0.25 ml. The incubation was carried out at 37°C in a water bath for 15 min. The reaction was terminated by the addition of 4.25 ml of a mixture of methanol, chloroform and water (2:2:0.25, v/v). Lipids were extracted according to [23] and separated by two-dimensional TLC in the presence of appropriate standards on silica gel plates. The first dimension was: chloroform/

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Abbreviations: cADPr, cyclic ADP-ribose; ER, endoplasmic reticulum; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; $InsP_3$, inositol 1,4,5-trisphosphate; MAM, mitochondria associated membranes; PKC, protein kinase C; PS, phosphatidylserine; TCA, trichloroacetic acid

0014-5793/00/$20.00
PII: S0014-5793(00)02058-5

methanol/ammonium hydroxide/water (55:33:4:2, v/v), the second dimension: chloroform/methanol/acetic acid/water (30:15:6:1, v/v). Phosphatidylserine was visualised with ninhydrin and scraped off for radioactivity counting.

2.4. Trypsin treatment

Trypsin treatment was performed at 20°C at a trypsin to membrane protein ratio of 1:1. The reaction was terminated by the addition of soybean trypsin inhibitor (ratio of trypsin inhibitor to trypsin, 2:1, w/w) [24]. After digestion, samples were assayed for enzyme activity without washing. In control incubations, trypsin inhibitor was added to the incubation mixture before trypsin.

2.5. Assays for marker enzymes

All fractions were analysed for NADPH-cytochrome *c* reductase determined by monitoring the reduction of cytochrome *c* at 550 nm according to Crane at al. [25]. A G-6-P phosphatase assay, modified from that of Swanson [26], was performed in duplicate for 15 min at 37°C. The 0.5 ml medium contained 0.3 ml 0.1 M citrate, pH 6.5, 0.1 ml 0.1 M G-6-P, pH 6.5, and 0.1 ml rat liver fractions. After addition of 0.5 ml 10% TCA, and storing on ice for 5 min, the samples were cleared by centrifugation, and aliquots of the supernatant were assayed for inorganic phosphate liberation at 660 nm. NAD pyrophosphorylase activity was determined by monitoring spectrophotometrically the formation of NADH at 340 nm as described [27,28]. 5′-Nucleotidase activity was measured according to the method of Howell and Palade with Triton X-100 omitted [29]. Liberated inorganic phosphate was measured at 660 nm.

3. Results

3.1. Serine base-exchange reaction in isolated nuclei

Rat liver nuclei used in this study were devoid of microsomes, mitochondria associated membranes (MAM) and plasma membrane contaminants. This was confirmed by measuring the activity of suitable marker enzymes. NADPH-cytochrome *c* reductase activity, a preferred marker for the ER, amounted in the nuclei fraction only to 0.48% of the activity found in the total liver homogenate (Table 1). Similarly, G-6-P phosphatase activity, microsomal as well as the MAM fraction marker enzyme, in the nuclei was less than 0.06% of the homogenate activity (Table 1). Because serine base-exchange activity has been observed in the plasma membranes of some cells [30], 5′-nucleotidase activity, the marker enzyme of this membrane, was also assayed herein. However, the activity of this enzyme in the nuclei amounted again only to 0.27% of the activity found in the homogenate (not shown). These determinations verified that the isolated nuclei were only slightly contaminated by the ER, MAM and plasma membrane subcellular fractions.

In such prepared nuclei, the Ca^{2+}-dependent serine base-exchange activity was assayed. The total activity of the enzyme, measured by [^{14}C]serine incorporation into PS, amounted to 1.3 nmol/min (4.26%), as compared to 30.2 nmol/min found in the homogenate, regarded as 100% (Table 1). The specific activity of serine base-exchange enzyme in the microsomes, the main locus of PS synthesis, amounted to 71.7 pmol/min/mg protein, whereas in the nuclei it amounted to 31.8 pmol/min/mg protein. These data confirm that isolated rat liver nuclei are the organelles in which Ca^{2+}-dependent PS synthesis takes place.

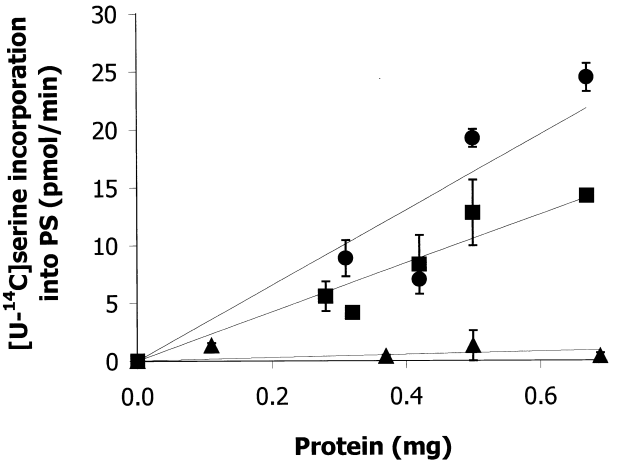

Fig. 1. Protein dependence of serine base-exchange activity in nuclei (■), nucleoplasts (●) and outer membrane of the nuclear envelope (▲). The points show the mean ± S.D. for 4–10 measurements.

3.2. Nucleoplasts are the site of PS synthesis

To obtain the outer nuclear membrane and nucleoplasts, a highly effective method of citrate treatment was used [19,31]. Fig. 1 shows the rate of [^{14}C]serine incorporation into PS depending on nuclear, nucleoplast and nuclear outer membrane protein concentrations. As shown, the highest [^{14}C]serine incorporation was observed in the nucleoplasts, whereas in the outer nuclear membranes the incorporation was negligible (Fig. 1). The specific activity of PS synthesis, calculated from the slope of the curves, amounted to 30.2 pmol/min/mg protein in nucleoplasts and 21.6 pmol/min/mg protein in the nuclei. These data show that the outer nuclear membrane, obtained by the citrate treatment of the nuclei, is not the site of PS synthesis.

3.3. Effect of trypsin on serine base-exchange activity in nucleoplasts

To examine localisation of the serine base-exchange in nucleoplasts we used the protease protection assay. This method is widely used for studying the sidedness of enzymes in various biological membranes, including nuclear membranes [24,31,32]. The susceptibility of serine base-exchange to pro-

Table 1
Purity of the nuclei isolated from rat liver homogenate and serine base-exchange activity

Fraction	Serine base-exchange		NADPH-cytochrome *c* reductase		Glucose-6-phosphate phosphatase	
	total activity (nmol/min) (%)	specific activity (pmol/min/mg protein)	total activity (μmol/min) (%)	specific activity (μmol/min/mg protein)	total activity (μmol/min) (%)	specific activity (μmol/min/mg protein)
Homogenate	30.2 ± 5.3 (100)	9.7 ± 2.2	13.9 ± 2.6 (100)	4.3 ± 0.50	271 ± 33 (100)	66.5 ± 8.2
Microsomes	19.0 ± 7.1 (63)	71.7 ± 17.1	9.6 ± 2.9 (69)	45.2 ± 6.9	50 ± 28 (18.6)	187.7 ± 83.6
Nuclei	1.29 ± 0.23 (4.26)	31.8 ± 8.6	0.07 ± 0.01 (0.48)	2.16 ± 0.50	0.17 ± 0.04 (0.06)	3.96 ± 1.15

Rat liver homogenate, microsomes and nuclei were isolated and enzyme activities assayed as described in Section 2. Figures in parentheses represent the percentage. Results are expressed as the means ± S.D. of five independent preparations.

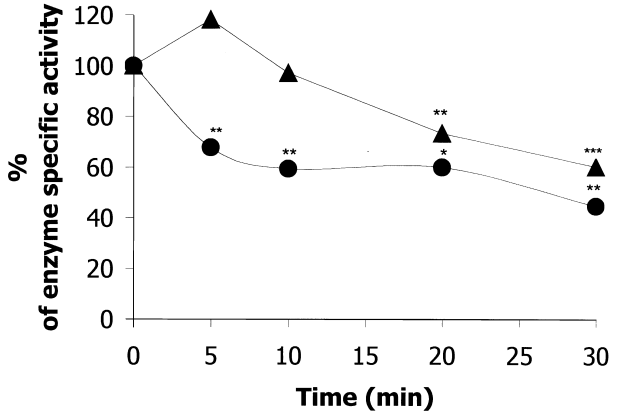

Fig. 2. Effect of trypsin digestion on serine base-exchange and NAD pyrophosphorylase activities in nucleoplasts. Nucleoplasts were preincubated with trypsin for the indicated time and immediately assayed for serine base-exchange (●) or NAD pyrophosphorylase (▲) activities. The enzyme activity of samples to which trypsin inhibitor was added before trypsin was taken as 100% and amounted to 24.6 ± 7.3 pmol of [U-^{14}C]serine incorporated/min/mg of protein for serine base-exchange, and 9.8 ± 0.1 nmol NADH/min/mg to NAD pyrophosphorylase. Data are presented as mean values ± S.D. for serine base-exchange activity ($n = 12$) and for NAD pyrophosphorylase ($n = 4$). Asterisks indicate statistical significance of differences estimated by Student's t-test: $*P < 0.1$; $**P < 0.01$; $***P < 0.001$.

teolytic digestion was investigated in experiments in which nucleoplasts were incubated for a fixed time (5 min) with increasing amounts of trypsin. These experiments showed that 1 mg of trypsin per 1 mg of nucleoplast protein produced 50% of enzyme inhibition (not shown). The enzyme activities of the samples to which trypsin inhibitor was added were taken as 100%. The trypsin-caused inhibition of the serine base-exchange reaction in nucleoplasts was compared to that of NAD pyrophosphorylase, known as a marker enzyme of the inner nuclear membrane [19] (Fig. 2). The data presented in Fig. 2 demonstrate that 5 min incubation of nucleoplasts with trypsin produced about 40% inhibition of the serine base-exchange activity. Such an inhibitory effect was maintained for 20 min incubation. In contrast, inhibition of NAD pyrophosphorylase during the first 5 min of trypsin digestion was negligible, and after 20 min of incubation amounted to about 25%. Thus, in the nucleoplasts, the serine base-exchange enzyme was susceptible for proteolysis, whereas NAD pyrophosphorylase was by far more resistant. These data may suggest that the serine base-exchange enzyme has an active site at the periplasmic side of the inner membrane of the nuclear envelope, whereas the catalytic centre of NAD pyrophosphorylase is most probably located at the internal, matrix, side of the nuclear envelope.

4. Discussion

The present study shows that PS is synthesised in rat liver nuclei. Moreover, our study demonstrates that this process occurs in the inner, and not in the outer, membrane of the nuclear envelope. These results are in favour of many studies which have revealed that, although the nuclear envelope outer membrane is continuous with surrounding ER (the main place of PS synthesis in the cell), these two membranes remain non-identical in their protein composition and many other properties [1–6].

PS synthesis in ER membranes is regulated by such signalling events that are responsible for calcium movement between cytoplasm and ER calcium stores [11–14]. The outer nuclear membrane contains the ER-type Ca^{2+}-pump ATPase and inositol 1,3,4,5-tetrakisphosphate receptors, which mediate nuclear calcium entry. The inner nuclear membrane contains inositol 1,4,5-trisphosphate ($InsP_3$) and ryanodine receptors that take part in calcium liberation into nuclear matrix [4]. $InsP_3$ as well as cyclic ADP-ribose (cADPr), are generated inside the nucleus. cADPr is synthesised in the inner nuclear membrane by ADP-ribosyl cyclase [31], whereas $InsP_3$ is produced as the result of phosphatidylinositol 4,5-bisphosphate hydrolysis via phospholipase C action [2,3]. The intranuclear generation of $InsP_3$ and cADPr could potentially activate their inner nuclear membrane receptors and trigger nucleoplasmic Ca^{2+} release [4,5,31]. It seems that this calcium movement should be able to regulate PS synthesis in the nucleus as it does in the ER.

The mitogen-induced nuclear diglycerides lead to the activation of nuclear PKC activity responsible for the phosphorylation of selected nuclear proteins [1,6]. For their full activity all PKC isoforms require PS [7]. Phosphatidylglycerol, a new activator of the nuclear PKC, described most recently, also needs PS for full activity of the enzyme [33]. All these events occur inside the nucleus. Thus, PS synthesis which occurs in the inner membrane of the nuclear envelope may be physiologically relevant.

In mammalian cells, two base-exchange enzymes synthesising PS have been identified: PSSI which exchanges serine, choline and ethanolamine, and PSSII, which does not exchange choline [8]. mRNA encoding these enzymes are differentially expressed in several murine tissues [34]. Immunochemical identification showed that PSSI is located mainly in the ER and in the MAM [35], whereas nuclei have never been tested in this respect. On the other hand, Stone and Vance described that in mouse liver, PSSI mRNA was especially abundant, whereas PSSII mRNA was not detected [34]. Furthermore, Albi and Viola-Magni reported the presence of the choline base-exchange activity in rat hepatocyte nuclei [36]. These data might suggest that PSSI is the type of enzyme which is present in rat liver nuclei, but the presence of PSSII could not be excluded.

In our previous study we have postulated that the base-exchange enzyme in the ER is a transmembrane protein containing the active site for serine accessible from the cytoplasmic surface of the ER and the active site for Ca^{2+} exposed at the luminal surface of the ER membrane [37]. The results of the present investigation indicate that the base-exchange enzyme present in the inner nuclear membrane has the domain important for the enzyme activity accessible from the periplasmic space of the nuclear envelope. Since this space is proposed to be a pool for nuclear Ca^{2+} [5], one can speculate that this domain could contain the active site for Ca^{2+}. Further studies are required to identify which type of PS-synthesising enzyme is present in the inner nuclear membrane and how it is situated.

Acknowledgements: The authors wish to thank Professor L. Wojtczak (Nencki Institute, Warsaw) for critical reading of the manuscript. This study was supported by a grant from the State Committee for Scientific Research (KBN, Poland) to the Nencki Institute.

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