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Nuclear Membrane Cholesterol Can Modulate Nuclear Nucleoside Triphosphatase Activity

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Abstract Previous work has suggested that changes in nuclear membrane cholesterol may induce a stimulation in nuclear nucleoside triphosphatase (NTPase) activity. The purpose of the present study was to directly investigate if nuclear membrane cholesterol can stimulate nuclear NTPase activity. The cholesterol content of nuclei was altered with a liposomal methodology. The cholesterol content of nuclei isolated from hepatic tissue was relatively low in comparison to that typically exhibited by other membrane fractions. Because of this, it was difficult to further deplete the nuclear membrane of cholesterol, but we could successfully increase the cholesterol content after exposure to cholesterol-enriched liposomes. Nuclear NTPase activity was potently stimulated (~150–200% of control) by an increase in the nuclear membrane cholesterol content. The Vmax of the NTPase activity in the presence of ATP or GTP was significantly increased after cholesterol enrichment without altering the affinity of the enzyme for these moieties. Mg^{2+} dependency of NTPase activity was also altered by cholesterol incorporation into the nuclear membrane. Cholesterol enrichment of the nuclear membrane also left the nuclei more susceptible to damage by salt-induced lysis than control nuclei. Our results clearly demonstrate that the cholesterol content of the nuclear membrane will have significant, direct effects on nuclear integrity and NTPase activity.

Key words: cholesterol, NTPase, nuclear membrane, pore complex, hepatic tissue

Although we are beginning to learn more about the factors which regulate the pore complex [Agutter and Prochnow, 1994; Bischoff et al., 1994; Gerace, 1992; Newmeyer and Forbes, 1988; Pante and Aebi, 1993; Richardson et al., 1988], the relationship of the nuclear membrane itself to pore function is not understood as well. A biochemical approach to this problem is to examine the influence that the nuclear membrane has on nuclear nucleoside triphosphatase (NTPase) activity. The NTPase has been proposed to regulate the opening of the nuclear pore complex [Agutter and Prochnow, 1994; Agutter et al., 1976; Newmeyer and Forbes, 1988; Prochnow et al., 1994; Richardson et al., 1988; Schroder et al., 1986]. If the NTPase protein is situated on the laminar proteins under the nuclear membrane and thereby physically removed from the nuclear membrane, one might expect that the lipid composition of the nuclear membrane would have no effect on NTPase activity (and pore function). This hypothesis is not consistent with previous work from our laboratory which has suggested that nuclear cholesterol content can increase in livers from JCR:LA-corpulent rats and this may induce an elevation in nuclear NTPase activity [Czubryt et al., submitted b]. Membrane integrity in these nuclei was also found to be abnormally susceptible to salt-induced lysis. Although the membrane cholesterol content was suggested as the mechanism responsible for these changes, many other mechanistic interpretations are possible in an in vivo animal model of disease. It would be helpful to examine the direct interaction of cholesterol with nuclear function in an in vitro setting where the number of external variables is more controlled. Therefore, the purpose of the present study was to focus upon the effects on NTPase activity of in vitro enrichment of hepatic nuclei with cholesterol.

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EXPERIMENTAL PROCEDURES

Materials

All chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO).

Isolation and Modification of Nuclei

Isolated intact nuclei were obtained from the livers of male Sprague-Dawley rats weighing between 300 and 350 g by a modification of the protocol described by Gilchrist and Pierce [1993]. Nuclei were rapidly frozen in liquid nitrogen and stored at −85°C until required.

Phosphatidylcholine and phosphatidylcholine-cholesterol liposomes were prepared as described [Kutryk and Pierce, 1988]. The cholesterol-enriched liposomes were prepared at a starting ratio of 2:1 cholesterol:phospholipid (mol:mol), but the small unilamellar vesicles which were incubated with the nuclei actually contained cholesterol:phospholipid at a ratio of 1.5:1.0, which is near the upper limit for the incorporation of cholesterol into phospholipid vesicles [Chapman, 1984; Kutryk and Pierce, 1988]. Isolated purified nuclei were incubated for 16–18 h at 4°C in the presence of cholesterol/phosphatidylcholine (initially 2:1 mol/mol) or phosphatidylcholine liposomes. The liposome/nuclei mixtures were then centrifuged at 104,000*g* for 25 min to separate the nuclei from the liposomal vesicles. The nuclei pellet was washed once and then resuspended in 250 mM sucrose, 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin to obtain a final protein concentration of 0.5–1.0 mg/ml.

Measurement of the cholesterol content of nuclear membranes was carried out after lipid extraction in chloroform:methanol [Kutryk and Pierce, 1988] and determined by an enzymatic method [Sale et al., 1984]. Phospholipid phosphorus measurements were carried out [Bartlett, 1959] after lipid extraction.

Measurement of NTPase Activity

Nuclear NTPase activity was assayed as follows. Nuclei (0.5 mg/ml) were preincubated for 10 min at 30°C in 360 μl of buffer containing 250 mM sucrose, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, and various concentrations of total $MgCl_2$ required for specified free Mg^{2+} concentrations in the presence of 5 mM GTP or ATP. Nucleoside dependence of NTPase activity was performed in the presence of 1 mM free Mg^{2+}. Calculations of bound and free ligands were performed using the computer based LIGAND program of Fabiato and Fabiato [1979]. Stability constants used for Mg.ATP and Mg.EDTA were 1.9×10^4 and 4.764×10^5, respectively. NTPase activity was initiated by the addition of the nucleoside triphosphate to the preincubation solution. NTPase activity was quenched by the addition of 10% SDS. Inorganic phosphate was measured according to the method of Raess and Vincenzi [1980].

RESULTS

Purified isolated nuclei were incubated with phosphatidylcholine or cholesterol/phosphatidylcholine liposomes (cholesterol/phospholipid ratio initially of 2:1 mol/mol). The liposomal technique is a well-established method to alter the cholesterol content of a membrane [Kutryk and Pierce, 1988; Papahadjopoulos, 1970]. At the end of the incubation period, the nuclei were separated from the liposomal vesicles by centrifugation. The treated nuclei pellet was then washed and spun down once more in order to be certain of a complete separation of nuclear from liposomal vesicles. The phospholipid content of the nuclei did not increase significantly after liposome treatment (Table I). This would indicate that adherence of liposomes to the nuclei did not represent a problem. Furthermore, in separate experiments, [^{3}H] cholesterol-enriched liposomes transferred cholesterol to nuclei with a

TABLE I. Modification of the Cholesterol Content of Nuclear Membranes†

	Control	Phosphatidylcholine-treated	Cholesterol-treated
Cholesterol content (nanomoles per milligram of protein)	2.2 ± 0.3	2.3 ± 0.4	6.1 ± 1.2*
% of control		104 ± 7	275 ± 18*
Phospholipid content (nanomoles per milligram protein)	21.8 ± 3.4	20.1 ± 2.8	20.9 ± 3.2
% of control		94 ± 5	96 ± 6
N	8	8	6

†Nuclei were untreated (control) or incubated overnight with cholesterol-free phospholipid liposomes (phosphatidylcholine-treated) or with cholesterol/phospholipid liposomes (2:1 mol/mol). See Experimental Procedures for cholesterol and phospholipid quantification protocols. Values represent means ± SE.

*$P < 0.05$ vs. control.

2.5-fold greater efficiency than [^{14}C] cholesterol oleate-enriched liposomes. This preferential transfer of cholesterol vs. cholesterol oleate from the liposomes to the nucleus would again indicate that adherence of the liposomes was not a major problem. In a series of experiments (data not shown) in which we incubated nuclei for various times, we found optimal incorporation of cholesterol occurred at 16–18 h of incubation with a 2:1 mol/mol liposomal ratio of cholesterol to phospholipid. Cholesterol content of treated nuclei increased almost threefold over control values (Table I). Incubation of phosphatidylcholine liposomes (cholesterol/phospholipid ratio of 0:1 mol/mol) with nuclei did not alter the cholesterol or phospholipid phosphate content of the nuclear membrane (Table I).

NTPase activity was examined as a function of reaction time after the nuclei were enriched with cholesterol (Fig. 1). ATP hydrolysis increased in all groups as the reaction time progressed. Significant differences were observed between control or phosphatidylcholine liposome–treated groups and cholesterol-enriched nuclei. This significance was apparent at all time points measured. There were no significant differences, however, between control and phosphatidylcholine-treated nuclei.

ATP hydrolysis by control and cholesterol-enriched nuclear membranes was examined as a function of varying [ATP] (Fig. 2). There were significant differences between control and cholesterol enriched nuclei at each [ATP]. Cholesterol enrichment induced a significant change in the Vmax value for the enzyme without altering the Km for ATP (Table II).

NTPase activity was also examined in the presence of GTP as a substrate instead of ATP.

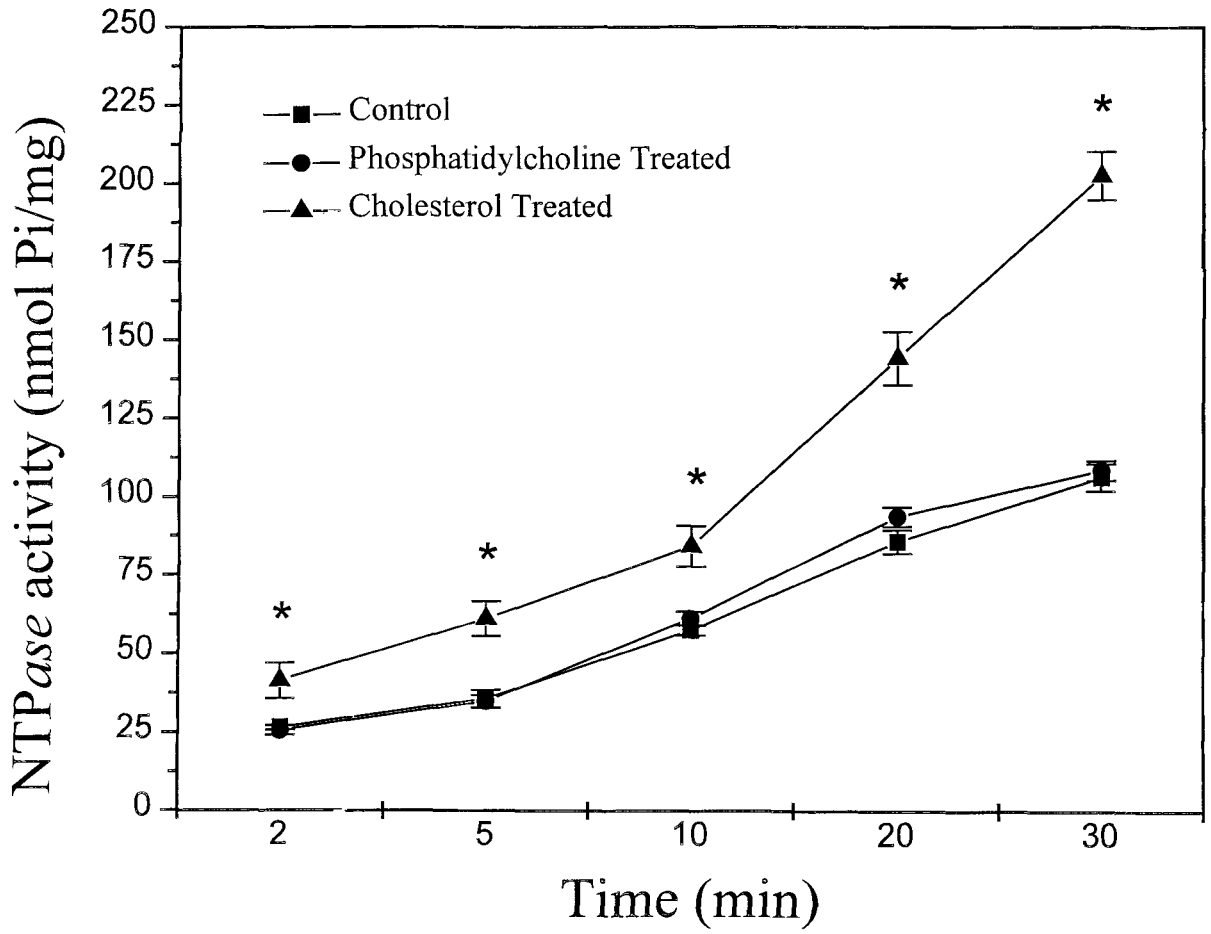

Fig. 1. Effect of cholesterol on NTPase activity of nuclear membranes as a function of reaction time. NTPase activity was initiated upon addition of ATP to a final concentration of 5 mM. Reactions were quenched at various times with 10% SDS. Significant differences are indicated (*$P < 0.05$) between control and cholesterol-enriched membrane preparations. Values represent means ± standard error measurements for six separate nuclear preparations.

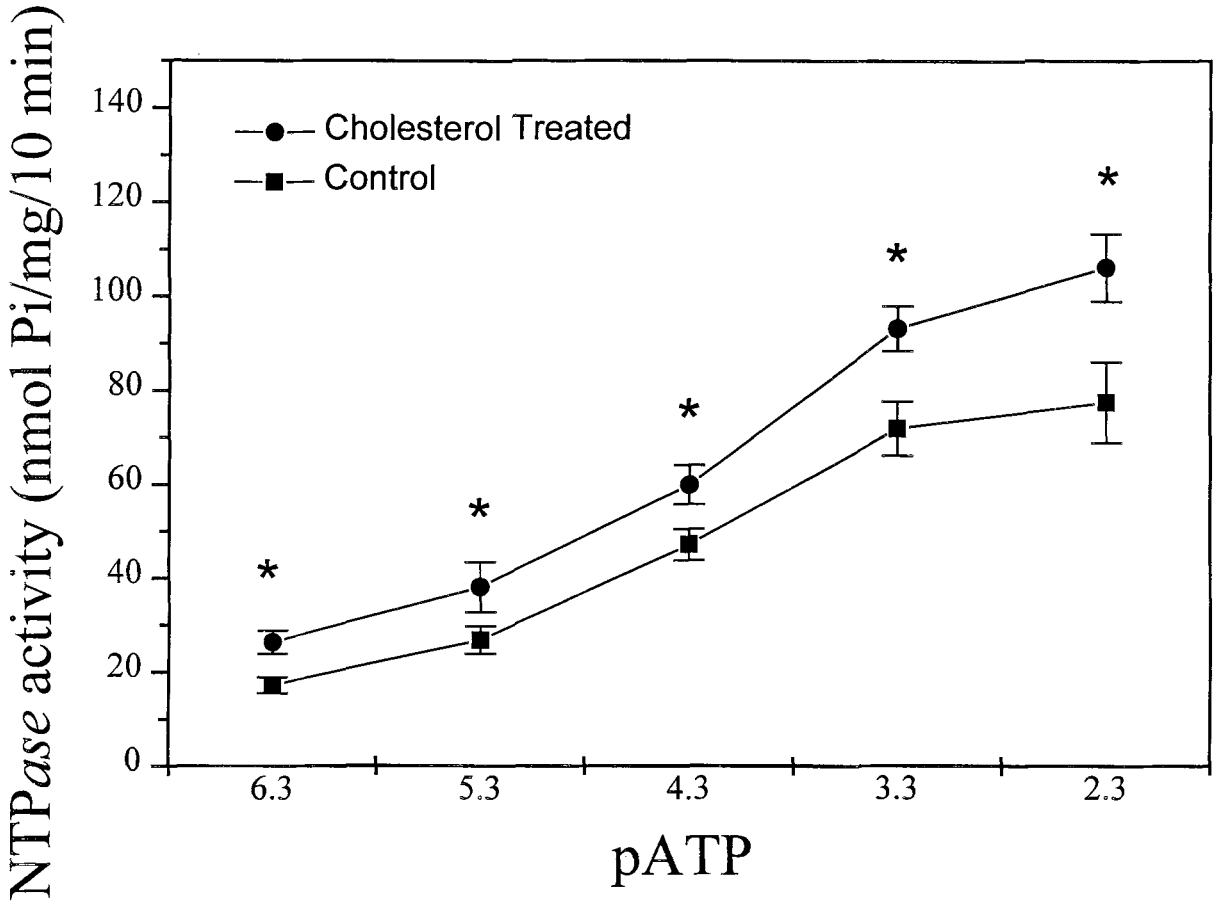

Fig. 2. NTPase activity of control and cholesterol-enriched nuclear membranes as a function of [ATP]. Reaction time was 10 min. Free Mg^{2+} was held constant at 1 mM. Significant differences (*$P < 0.05$) in NTPase activity are indicated between control and cholesterol-enriched preparations. Values represent means ± standard error measurements for six preparations.

TABLE II. Effect of Cholesterol Enrichment on the Kinetics of Nuclear Nucleoside Triphosphatase Activity†

	Control nuclei		Cholesterol-enriched nuclei	
	Km (μM)	Vmax (nmol/mg/10 min)	Km (μM)	Vmax (nmol/mg/10 min)
GTP	98.5 ± 5.3	107.6 ± 8.3	81.5 ± 7.4	141.7 ± 9.9*
ATP	91.3 ± 8.4	77.8 ± 5.4	83.6 ± 6.0	106.6 ± 7.2*

†Cholesterol enrichment was carried out as described in Experimental Procedures. Data were calculated from Hanes plots. Values represent means ± S.E. (n = 5–6).
*$P < 0.05$ vs. control values.

GTP*ase* activity of control and cholesterol-enriched nuclear membranes increased with time (Fig. 3). Significant differences were observed between control and cholesterol enriched membrane preparations (Fig. 3). GTP*ase* activity of control and cholesterol-enriched nuclear membranes was also studied as a function of [GTP] (Fig. 4). Significant differences were observed between control and cholesterol-enriched membrane preparations. Cholesterol enrichment of nuclei induced a significant change in the Vmax but not the Km of the enzyme in the presence of GTP (Table II).

The Mg^{2+} dependence of the enzyme activity of control, phosphatidylcholine-treated, and cholesterol-enriched nuclear membranes is shown

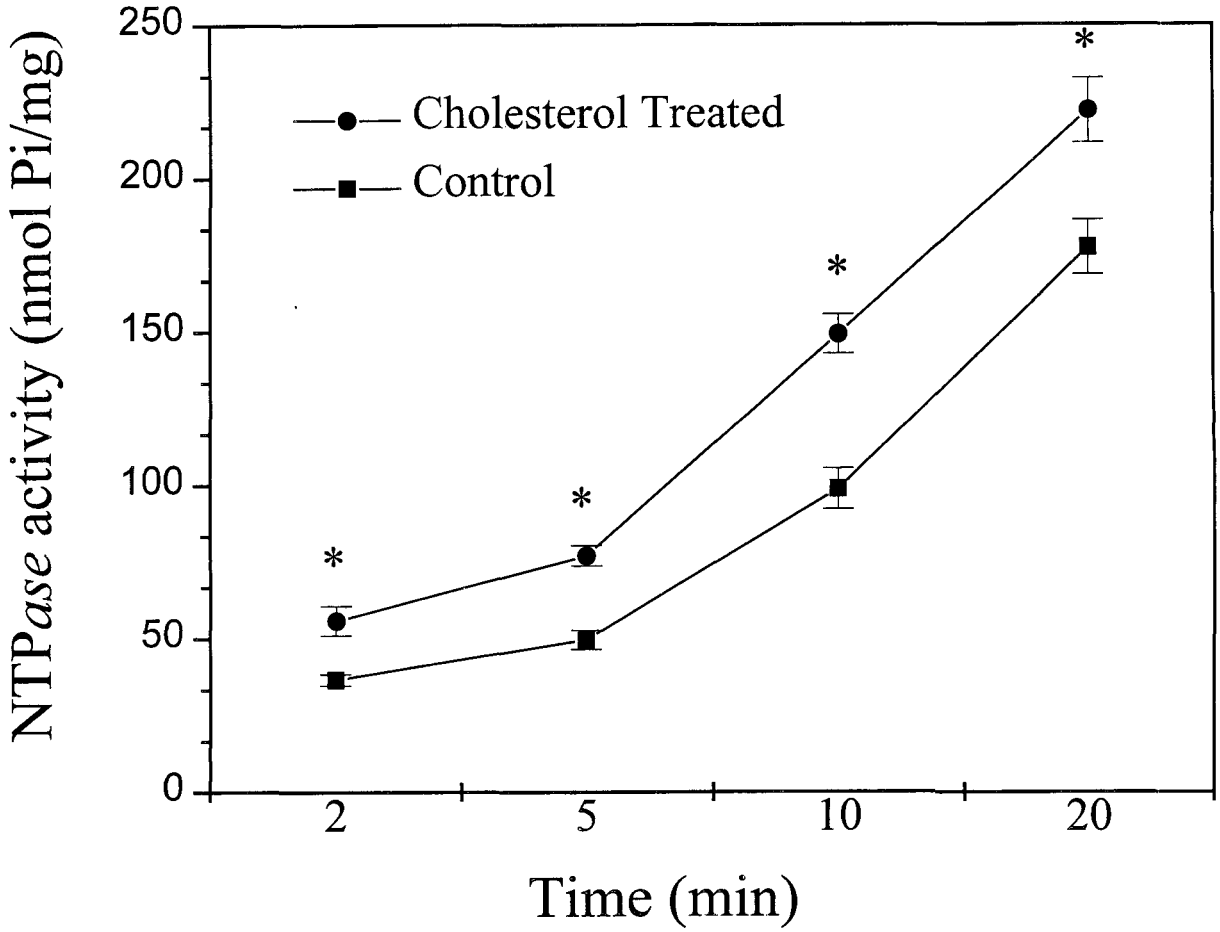

Fig. 3. NTPase activity as a function of time in control and cholesterol-enriched nuclear membranes in the presence of GTP. NTPase activity was assayed as in Fig. 1 using GTP as the substrate. Free Mg^{2+} was held constant at 1 mM. Significant differences in NTPase activity are indicated ($*P < 0.05$) between control and cholesterol-enriched preparations. Values represent means ± standard error measurements for five preparations.

in Figure 5. Significant differences in NTP*ase* activities were observed between control or phosphatidylcholine treated nuclei and cholesterol enriched preparations. Statistical significance was observed at various [Mg^{2+}]. Maximum stimulation of activity was observed at approximately 3.16 mM free Mg^{2+}. There was an rapid increase in enzyme activity as free [Mg^{2+}] was increased from 1.0 mM to 3.16 mM for control, phosphatidylcholine-, and cholesterol-treated nuclei. There were no significant differences between control and phosphatidylcholine-treated nuclei.

The relationship of enzyme activity to the cholesterol content of nuclei was plotted in Figure 6 from results obtained from a number of individual experiments. These data points include control and cholesterol-enriched membranes. A significant relationship was demonstrated for nuclear cholesterol content and membrane NTPase activity ($r = 0.817$; $P < 0.0001$).

The effect of cholesterol incorporation on nuclear membrane integrity was also studied. Release of nucleotides from the nuclei in response to in vitro salt-induced lysis was used as an indicator of nuclear membrane integrity [Czubryt et al.]. Cholesterol-enriched nuclei released more nucleotides at a similar [NaCl] than untreated or phosphatidylcholine-treated nuclei (Fig. 7). The concentration of NaCl at which 50% maximal nucleotide release was observed (RC_{50}) was also significantly lower in the cholesterol-enriched nuclei in comparison to the two control groups (Table III).

The relationship between nuclear membranes' susceptibility to disruption and that of membrane cholesterol content are depicted in Fig-

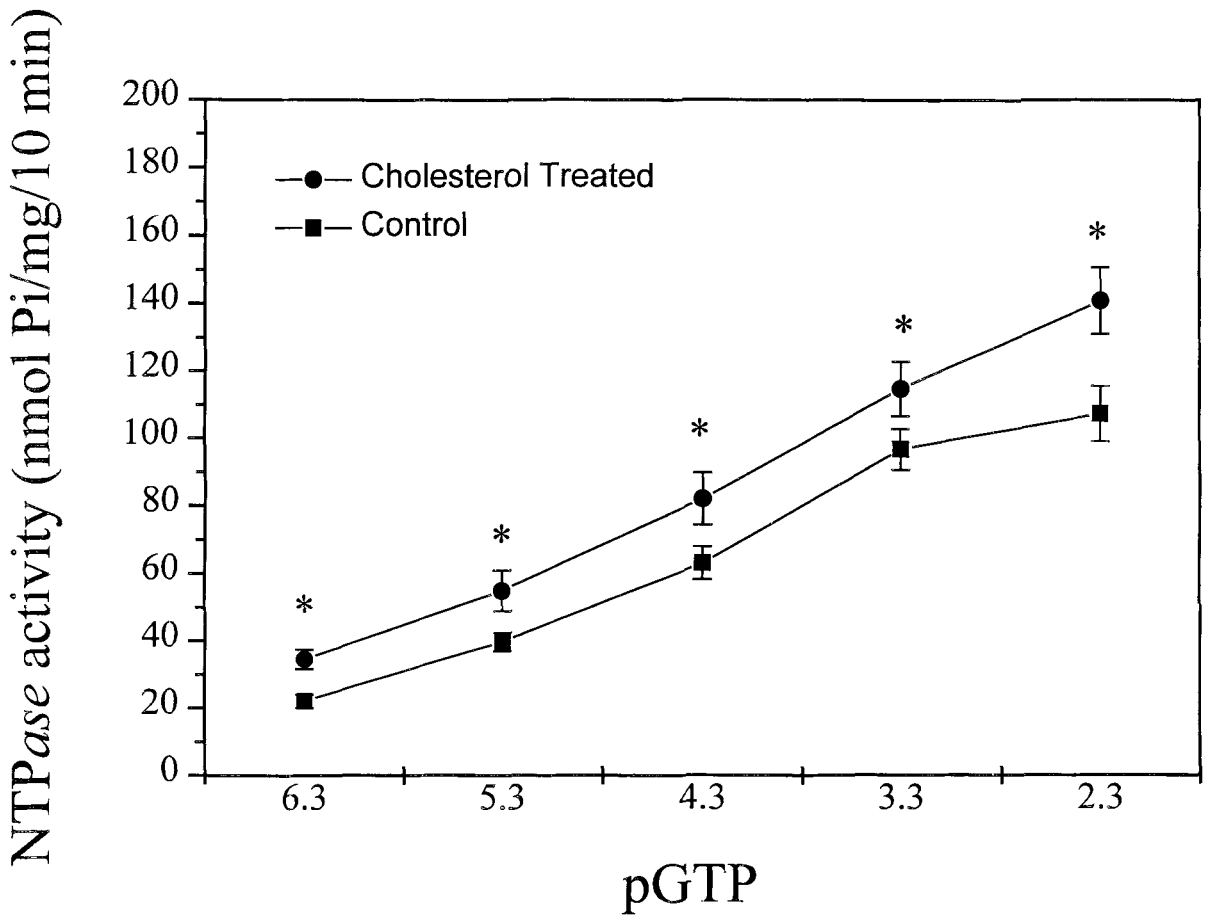

Fig. 4. NTPase activity of control and cholesterol-enriched nuclear membranes as a function of [GTP]. Reaction time was 10 min. Free Mg^{2+} was held constant at 1 mM. Significant differences in NTPase activity are indicated (*$P < 0.05$) between control and cholesterol-enriched preparations. Values represent means ± standard error measurements for five preparations.

ure 8. Data from this and a previous study [Czubryt et al., submitted b] involving in vivo elevation of cholesterol are plotted. The RC_{50} value in millimolar and cholesterol content in nanomoles per milligram of protein are plotted from both studies. An interdependent relationship exists ($r = -0.996$) between nuclear cholesterol content and nuclear disruption ($P = 0.004$).

DISCUSSION

Our results clearly demonstrate that the nuclear NTPase is sensitive to the cholesterol content of the nuclear membrane. The nuclear cholesterol content was 15–100-fold lower than values reported previously for plasma membranes, mitochondria, and sarcoplasmic reticulum [Ganguly et al., 1983; Kutryk and Pierce, 1988; Pierce and Dhalla, 1985]. This low cholesterol content left the nuclei difficult to deplete of cholesterol any further. Long (24–48 h) incubations with liposomes which did not contain cholesterol did not result in a significant reduction in nuclear cholesterol levels. The movement of cholesterol with the liposomal technique is via diffusion that is dependent upon concentration differences between the liposomes and the membrane [Chapman, 1984]. It is likely that the cholesterol concentration within the nuclei was insufficient to induce a significant movement of cholesterol out of the nuclear membrane. This in vitro finding would also suggest that if nuclear cholesterol is to be modified in vivo, it will be more likely to increase than to decrease. Work on nuclei obtained from hyperlipidemic rats supports this hypothesis [Czubryt et al., in press]. This study [Czubryt et al.] showed that the nuclear membrane cholesterol increased in vivo and the NTPase activity increased with it. The present data provide significant direct evidence that nuclear membrane cholesterol can modu-

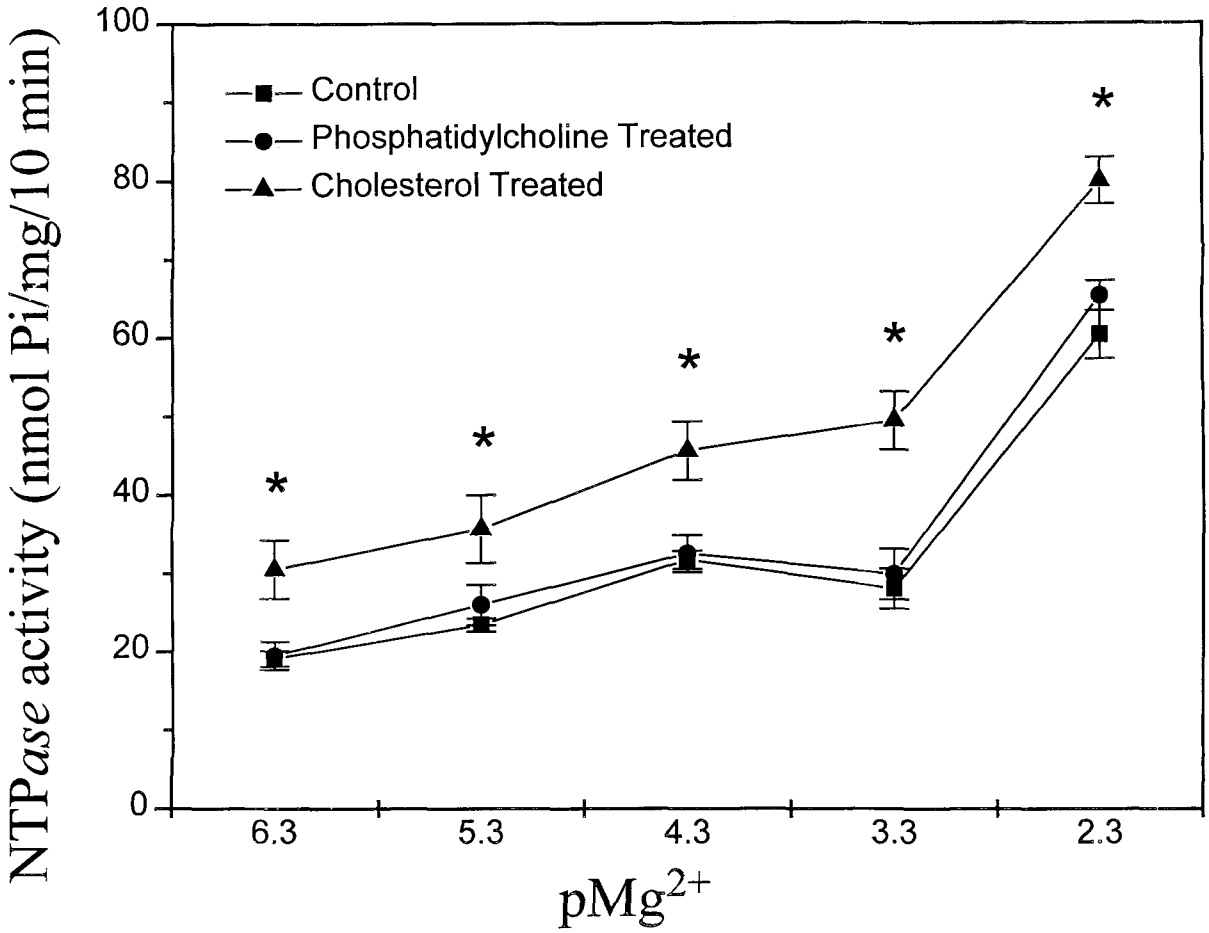

Fig. 5. NTPase activity of control, phosphatidylcholine-treated, and cholesterol-enriched nuclear membranes as a function of [Mg^{2+}]. NTPase activity was assayed as in Fig. 1. Reaction time was 10 min. ATP concentration was 5 mM. Significant differences in NTPase activity are indicated (*$P < 0.05$) between control and cholesterol-enriched preparations (n = 5).

late NTPase activity. It is also worth noting that the increase in nuclear cholesterol content previously observed in vivo [Czubryt et al., submitted b] is very similar to the increase achieved in vitro in the present study (250% vs. 275% of control, respectively).

Two possible mechanisms may be responsible for the effects of cholesterol on nuclear NTPase activity. Some enzymes are influenced by membrane cholesterol directly [Kutryk and Pierce, 1988; Luciani et al., 1991]. The cholesterol may be in close proximity to the enzyme imbedded in the membrane, and this may have a direct effect on enzyme function. Alternatively, cholesterol is known to affect other enzymes in an indirect manner by altering the lipid fluidity in a general way throughout the membrane [Yeagle, 1989]. Increases in membrane cholesterol can increase membrane rigidity (or decrease fluidity) in a sphingomyelin-poor membrane like the nuclei, or, conversely, depletion of the cholesterol content can increase the lipid fluidity of the membrane [Corvera et al., 1992; Strubbs and Smith, 1984]. This change in the membrane's biophysical properties can dramatically affect enzyme function [Brasitus et al., 1988; Rotenberg and Zakim, 1991; Yeagle, 1989]. The direction of this change (stimulation or inhibition of enzyme activity) is entirely dependent upon the enzyme examined. Some enzymes are stimulated by cholesterol enrichment in the same membrane that other enzymes are inhibited [Kutryk and Pierce, 1988]. The stimulation of nuclear NTPase activity by cholesterol in the present study probably represents an indirect effect of cholesterol on the enzyme. This conclusion is based upon the location of the NTPase within the nucleus. The NTPase catalytic site appears to reside as part of a laminar protein subcomponent [Clawson et al., 1988; Tong et al., 1993]. The laminar pro-

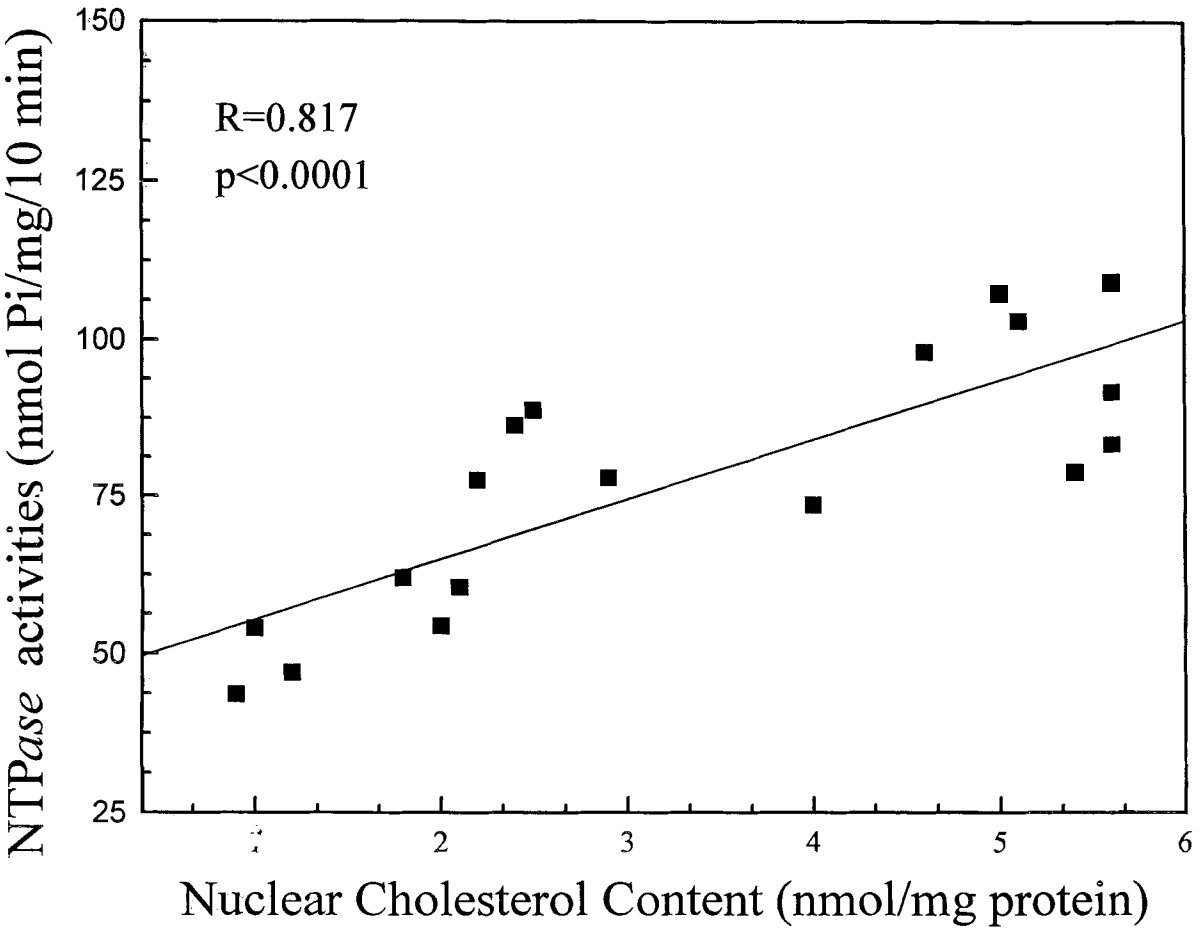

Fig. 6. NTPase activity as a function of nuclear membrane cholesterol content. The results from 18 experiments relating NTPase activity to the nuclear membrane cholesterol content are depicted here. NTPase activity exhibited a significant correlation with cholesterol content ($r = 0.817$; $P < 0.0001$). NTPase activity was measured for 10 min in the presence of 5 mM ATP.

teins form a cytoskeletal mesh along the inner surface of the nuclear membrane [Gerace and Burke, 1988; Konstantinov et al., 1995; Nigg, 1992]. The NTPase activity is dependent upon a hydrophobic environment but does not rely upon the bulk phospholipid content of the membrane [Smith and Wells, 1984]. Without evidence, therefore, of the NTPase imbedding itself into the membrane bilayer in a conventional manner, it is difficult to imagine a cholesterol annulus forming around the enzyme resulting in a requirement of the enzyme for cholesterol. Alternatively, it is more likely that changes in membrane cholesterol alter membrane fluidity and consequently how proteins like the NTPase interact with the membrane. It is relevant to emphasize that others have shown that changes in the membrane fatty acid composition of the nuclei alters NTPase activity and mRNA transport [Venkatraman and Clandinin, 1988; Venkatraman et al., 1986]. This was suggested to occur through a modulation of the physical properties of the membrane. More directly, another study has shown that nuclear membrane lipid fluidity has important effects on nucleocytoplasmic RNA transport [Herlan et al., 1979]. It is not unreasonable to hypothesize, therefore, that the incorporation of cholesterol into the nuclear membrane in the present study alters NTPase activity via a change in membrane rigidity.

The functional and pathophysiological implications of the present study are twofold. First, the increased nuclear cholesterol content may challenge membrane integrity. The response of cholesterol-enriched nuclei to increasing [NaCl] suggests that cholesterol incorporation has left the membrane integrity more susceptible to damage from stressful stimuli. This observation strengthens previous conclusions that cholesterol enrichment of the nuclei from JCR:LA-cp

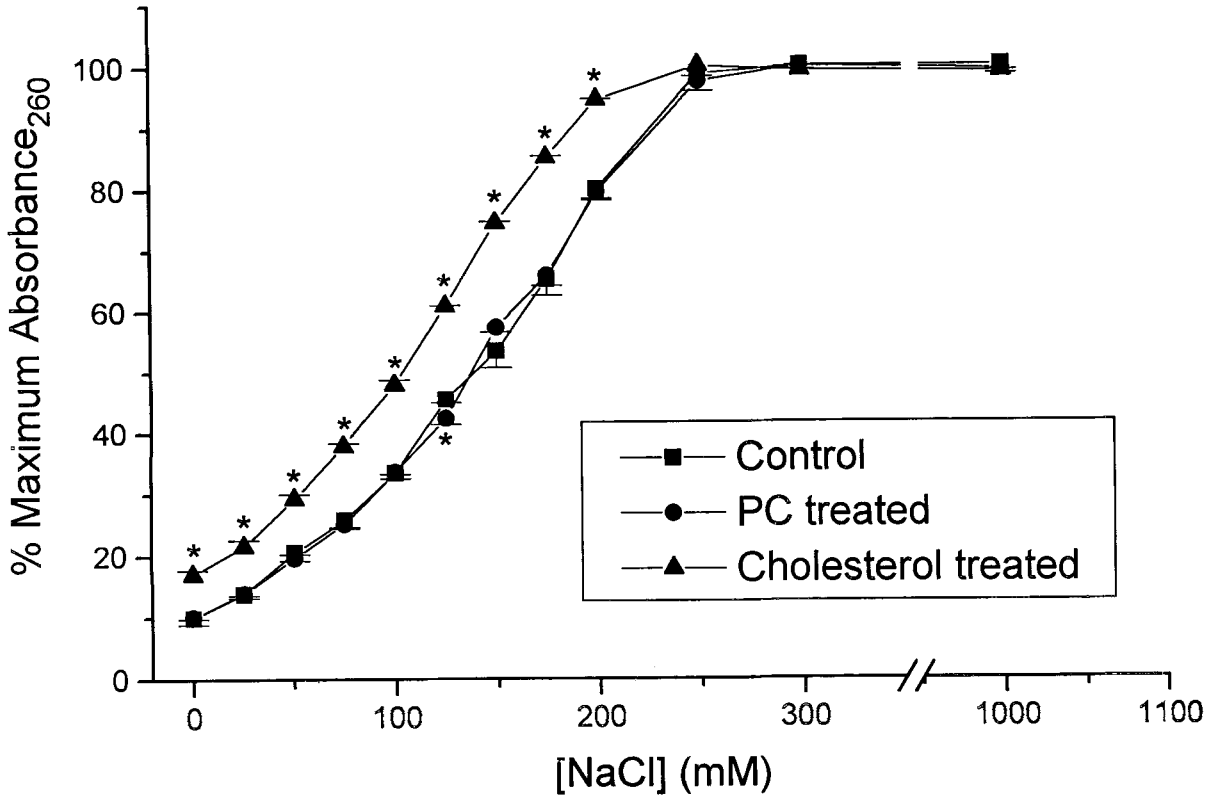

Fig. 7. Normalized A_{260} in supernatants from nuclei after treatment with increasing [NaCl]. Significant differences (*$P < 0.05$) are indicated between control, cholesterol-enriched, and phosphatidylcholine-treated (PC) nuclei. Values represent means ± standard error measurements for three preparations.

TABLE III. Effect of Cholesterol Enrichment on the Integrity of Isolated Nuclei†

	RC_{50} (mM)
Control	143 ± 6
Phosphatidylcholine	138 ± 1
Cholesterol	104 ± 1*

†The NaCl concentration (millimolar) at which the intact nuclei population released 50% of their maximal nucleotide content due to salt-induced lysis was measured. Nuclei from control (untreated), cholesterol-free (phosphatidylcholine-treated), or cholesterol-enriched (cholesterol-treated) preparations were tested. Values represent means ± S.E.
*$P < 0.05$ vs. control; $P < 0.05$ vs. phosphatidylcholine-treated.

rats was responsible for the increased susceptibility to salt-induced lysis [Czubryt et al., submitted b]. Indeed, plotting the relationship between nuclear cholesterol content and the RC_{50} value for preparations from the present study and those observed in nuclei from JCR:LA-cp rats [Czubryt et al., submitted b] demonstrates a strong association between the two parameters (Fig. 8) ($r = -0.99$). Secondly, and more importantly, is the relevance of the changes in NTPase activity to nucleocytoplasmic transport. Translocation of mRNA from the nucleus into the cytoplasm occurs in an energy-dependent manner through the nuclear pore complex [Dargemont and Kuhn, 1992; Garcia-Bustos et al., 1991]. The nuclear NTPase may be responsible for the gating of the nuclear pore complex [Agutter and Prochnow, 1994; Agutter et al., 1976; Newmeyer and Forbes, 1988; Prochnow et al., 1994; Richardson et al., 1988; Schroder et al., 1986]. Alterations in NTPase activity result in proportionate changes in mRNA flux through the pore complex [Agutter et al., 1976; Clawson et al., 1980; Prochnow et al., 1994]. Based upon these previous observations, it is reasonable to propose, therefore, that the changes in NTPase activity induced by cholesterol in the present study will have significant effects on mRNA translocation and nuclear pore function in general.

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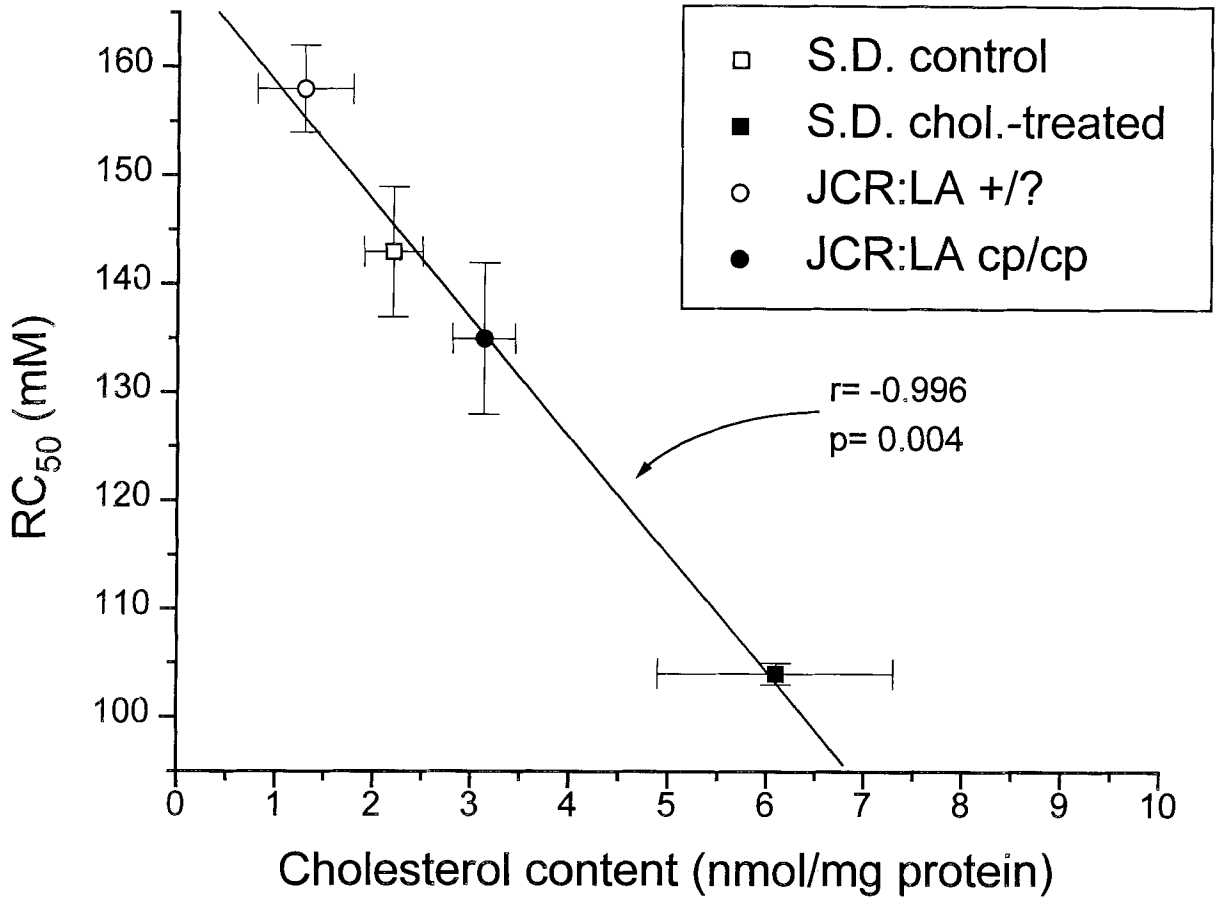

Fig. 8. Relation of nuclear membrane cholesterol content and susceptability to rupture due to salt-induced lysis. The RC_{50} (millimolar) and cholesterol content (nanomoles per milligram of protein) are plotted for control (open box) and cholesterol-enriched (solid box) nuclei in the present study and JCR:LA-cp lean (open circle) and corpulent (solid circle) nuclei from previous work [Czubryt et al., submitted b]. Values represent means ± standard error measurements for seven to eight preparations. A strong correlation ($r = -0.996$) exists between nuclear cholesterol content and the RC_{50} value ($P = 0.004$).

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