5′-Nucleotidase is activated upon cholesterol-depletion of liver plasma membranes

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The cholesterol content of rat liver plasma membranes was manipulated using either cholesterol-free or cholesterol-enriched liposomes. Removal of cholesterol from the membranes led to a marked increase in 5′-nucleotidase activity. However, increase in cholesterol content failed to exert any significant effect on 5′-nucleotidase activity. Arrhenius plots of the activity of the native enzyme exhibited a break at around 28°C with the activation energy of the reaction less above this temperature than below. In cholesterol-depleted membranes a single break at around 26°C was observed with activation energies greater above this temperature than below it. In cholesterol-enriched membranes Arrhenius plots were linear over the range examined. It is suggested that the lipid environment of the external half of the bilayer only influences 5′-nucleotidase activity in these membranes and that cholesterol exerts controlling effects on both the activity and conformation of the enzyme in native membranes.

5′-Nucleotidase *Cholesterol* *Plasma membrane* *Lipid–protein interaction*

1. INTRODUCTION

5′-Nucleotidase is an integral glycoprotein of the rat liver plasma membrane [1–5] with its active site exposed at the external cell surface [4–7]. Its activity appears to be particularly sensitive to the lipid environment. This is reflected by the occurrence of breaks in Arrhenius plots of its activity which correspond to temperatures where lipid phase separations have been identified in the membranes [8–13]. Indeed the membrane-bound enzyme appears to be particularly sensitive to activation elicited by increases in bilayer fluidity achieved either by the neutral local anaesthetic, benzyl alcohol [14] or by anionic drugs [15]. On the other hand, the activity of the solubilized enzyme, which is removed from the constraints of its membrane environment, is unaffected by such agents [16]. However, breaks in the Arrhenius plots of the detergent-solubilized enzyme can be observed. These correspond to micellar re-arrangements, in the detergent–lipid–5′-nucleotidase complex, occurring at the melting/pouring-point of the detergent [16], again signifying the sensitivity of the activity of this enzyme to alterations in its immediate environment. The activity of this enzyme may be influenced by lipids of the external half of the bilayer only in rat liver plasma membranes [15]. This is based upon observations that, of drug which can exert selective perturbations of the outer and inner halves of the bilayer, only those drugs exerting effects on the outer half influence 5′-nucleotidase activity.

We have developed methods [17,18] to manipulate the cholesterol content of isolated rat liver plasma membranes in vitro. This is carried out at low temperature to minimise any denaturation of the enzymes and under conditions whereupon exchange of cholesterol occurs between exogenous liposomes and the plasma membranes. Using this procedure we have shown that the activity of adenylate cyclase is optimal at native cholesterol/phospholipid ratios [18,19]. Either elevation or depression of the cholesterol content

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leads to enzyme inhibition. Here we demonstrate the effect of manipulating the cholesterol content of liver plasma membranes on 5'-nucleotidase activity.

2. MATERIALS AND METHODS

All biochemicals were from Sigma except for cholesterol oxidase which was from Boehringer (UK). All other chemicals were from BDH (Poole). Radiochemicals were from Amersham (Bucks).

Rat liver plasma membranes were prepared from male Sprague-Dawley rats (200–300 g) and stored as in [20]. The assay of 5'-nucleotidase was performed radiometrically [5]. The final concentration of 5'-AMP in the assay was 1 mM, which was saturating under all conditions [16]. Under all conditions initial rates were measured and final pH kept constant at pH 7.4.

Methods of cholesterol manipulation of liver plasma membranes were performed exactly as described in considerable detail in [17,18]. Briefly, this involved incubating at 4°C, membranes with either liposomes consisting of dipalmitoyl phosphatidyl choline (DPPC) to deplete membrane cholesterol, or with DPPC–cholesterol liposomes (cholesterol/phospholipid (C/P) molar ratio of up to 2.1–2.6) to elevate membrane cholesterol. By incubating membranes for various periods of time with liposomes of various cholesterol/phospholipid ratios it was possible to manipulate membrane cholesterol/phospholipid ratios and monitor changes in 5'-nucleotidase activity. An incubation mixture was designed [17,18] to prevent fusion of the exogenous liposomes with the membranes. This was checked using radiolabelled DPPC [17,18]. Methods of lipid analysis were performed as in [17,18].

3. RESULTS

Native liver plasma membranes have a cholesterol/phospholipid (C/P) molar ratio of 0.71 ± 0.02 (mean ± SEM, n = 11, see [18]). Incubation of liver plasma membranes at 4°C for up to 6 h with liposomes having a C/P ratio of 0.7 failed to exert any effect (<5%) on their 5'-nucleotidase activity. No change in activity was observed over this period when incubations were carried out in the absence of liposomes, showing that the 5'-nucleotidase was stable to prolonged incubation at 4°C in the incubation mixture. Indeed, incubation of membranes with the cholesterol-enriched DPPC liposomes (C/P = 2.1–2.6) failed to elicit alterations in 5'-nucleotidase activity (fig.1) even though the C/P ratio of the membranes was increased from 0.71 to around 0.98. However, incubation of membranes with DPPC liposomes alone caused a marked reduction in the cholesterol content of the membranes, from C/P = 0.71 to C/P = 0.32 [19]. This was accompanied (fig.1) by a dramatic increase in 5'-nucleotidase activity, which was some 2.5-fold greater than that of the native enzyme when a cholesterol/phospholipid ratio of 0.32 was attained.

Arrhenius plots of 5'-nucleotidase activity in cholesterol-enriched membranes (C/P ratio = 0.94–0.98) appeared to be linear over the temperature range examined, exhibiting an activation energy of 74.2 ± 15.5 kJ.mol^{-1} (SD, n = 3).

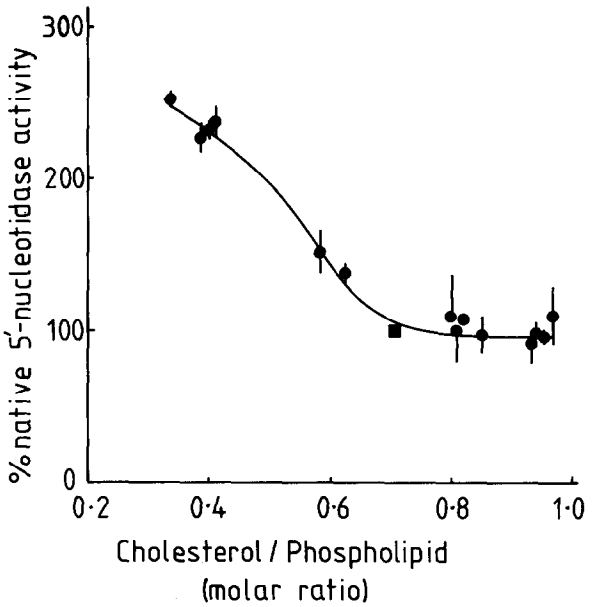

Fig.1. The influence of cholesterol on the 5'-nucleotidase activity of liver plasma membranes. Assays were carried out at 30°C on liver plasma membranes whose cholesterol content had been manipulated by incubation with either cholesterol-rich or cholesterol-free dipalmitoyl phosphatidyl choline liposomes [17,18]. Activity is expressed as a percentage of that exhibited by native membranes which had a C/P molar ratio of 0.71. Errors are SD (n = 3).

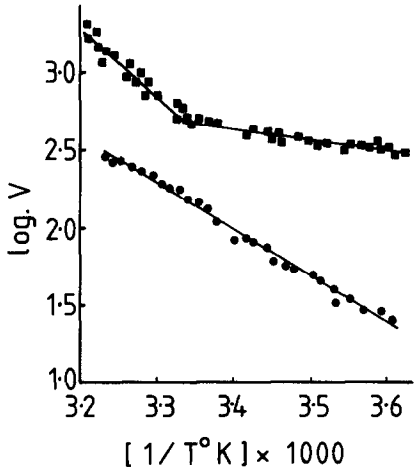

Fig.2. Arrhenius plots of 5′-nucleotidase activity in cholesterol-depleted and cholesterol-enriched liver plasma membranes. Initial rates were measured over the entire temperature range studied using cholesterol-enriched (●) membranes (C/P = 0.94) and cholesterol-depleted (■) membranes (C/P = 0.34).

In contrast Arrhenius plots of 5′-nucleotidase activity in cholesterol-depleted membranes (C/P ratio = 0.34–0.40) were clearly biphasic with a break point occurring at 26.8 ± 0.3°C and activation energies for the reaction of 112.9 ± 19.5 kJ.mol^{-1} above the break and 28.5 ± 7.6 kJ.mol^{-1} below the break (SD, n = 3). These break points contrast with the enzyme in native membranes which exhibits a break at around 27.5°C with activation energies of 46.4 ± 2.5 kJ.mol^{-1} above the break and 94.5 ± 13.4 kJ.mol^{-1} below the break (n = 5, [15]).

4. DISCUSSION

The methods employed allowed the cholesterol content of liver plasma membranes to be manipulated without affecting the phospholipid composition of the membrane [18,19]. Furthermore, 5′-nucleotidase activity was shown not to be affected either by prolonged incubation at 4°C or by incubation with DPPC-cholesterol liposomes of a C/P ratio similar to that of the native membranes. Interestingly however, elevation of the cholesterol content of liver plasma membranes failed to exert any noticeable effect on 5′-nucleotidase activity (fig.1). This was despite the fact that such a change in cholesterol content of the membranes led to a considerable decrease in bulk bilayer fluidity, equivalent to a change effected by a 6°C decrease in temperature [18]. This, at first sight, appears to be somewhat surprising as increases in bilayer fluidity, of a similar magnitude, achieved by local anaesthetics [14,15] elicit a significant activation of this enzyme. One possible explanation for this is that 5′-nucleotidase might exist in a relatively rigid microenvironment which would make it somewhat insensitive to any decreases in the fluidity of the bulk lipid phase. In this respect it is of interest to note that when the enzyme was purified from liver plasma membranes a single phospholipid, sphingomyelin remained tightly associated with 5′-nucleotidase [13,21,22]. In liver plasma membranes this lipid is rather unusual [23] in that not only are its associated acyl chains almost entirely saturated but most of them are particularly long. Thus if sphingomyelin is closely associated with the enzyme in these membranes it would be expected to provide a rather rigid environment for 5′-nucleotidase. Furthermore, cholesterol has been demonstrated to exhibit a particular preference for interaction with sphingomyelin [24] over other phospholipids. This could explain why cholesterol is also detected [12] in the purified 5′-nucleotidase–sphingomyelin complex. It is not too unlikely then that the microenvironment of the enzyme in the membrane has a relatively high cholesterol and sphingomyelin content compared with bulk lipid pool. If, at native C/P ratios the sphingomyelin environment of the enzyme is essentially saturated with cholesterol then any increases in the cholesterol content of the membrane would be expected to exert little effect. However, the lipid phase separation occurring at around 28°C in native membranes is either abolished or shifted to a temperature outside the range studied (5–35°C) by increase in the C/P ratio to about 0.98 [17]. Clearly 5′-nucleotidase is not entirely insensitive to events occurring in the bulk lipid pool as Arrhenius plots of its activity in native membranes exhibit a break at around 28° [9], yet this appears to be abolished (fig.2) in membranes with an elevated cholesterol content. We suggest here that the immediate lipid environment of 5′-nucleotidase exerts a dominating effect on its activity, a situation similar to that proposed for the Ca^{2+}-ATPase

from rabbit muscle sarcoplasmic reticulum [25–27].

Cholesterol-depletion of liver plasma membranes leads to the surprising observation that bilayer fluidity, as detected by a nitroxide-labelled fatty acid, appears to actually decrease [18]. However, there is evidence to suggest that the liver plasma membrane has 'cholesterol-rich' and 'cholesterol-poor' domains, where the fatty acid spin probe selectively monitors the 'cholesterol-poor' domains (see [18] for discussion). Presumably the apparent decrease in fluidity observed, upon cholesterol-depletion, was due to the release of the rather rigid sphingomyelin into the domains monitored by the fatty acid spin probe. 5′-Nucleotidase activity was however strikingly increased upon cholesterol removal from the membrane (fig.1). It is tempting to suggest that this was due to either an increase in the fluidity of the microenvironment of the enzyme caused by cholesterol depletion or due to the loss of a direct inhibitory effect exerted by cholesterol on the enzyme. Again these results suggest that the lipid microenvironment of the enzyme exerts a dominating effect on its activity. Certainly, cholesterol appears to have a potent regulatory effect on 5′-nucleotidase as indicated by this enhanced activity occurring upon cholesterol removal and also by the dramatic change in the form of Arrhenius plots of 5′-nucleotidase activity. In native membranes there is a break at around 28°C, with activation energies greater below the break than above it [9,15]. However, in cholesterol-depleted membranes, although a break occurs at around 27°C, activation energies are greater above the break point than below it (fig.2), suggesting that cholesterol exerts effects on the conformation of the protein. Indeed, we have noticed that a similar change in the form of the Arrhenius plots of 5′-nucleotidase activity occurs using the enzyme from weanling animals, where the membrane cholesterol content would be expected to be slowly increasing to adult concentrations [28]. This implies that cholesterol may be an important regulator of the functioning of this protein.

Arrhenius plots of 5′-nucleotidase activity in cholesterol-depleted membranes do not however exhibit any break at around 13°C, even though a lipid phase separation has been detected occurring in the membranes at this temperature [18]. However, we have suggested that in cholesterol-depleted membranes the lipid phase separation occurring at around 25°C is localized to the external half of the bilayer, whereas that at 13°C is localized to the inner half of the bilayer. That 5′-nucleotidase experiences only the higher-temperature lipid phase separation provides further support for our contention [16] that in liver plasma membranes the activity of 5′-nucleotidase is influenced only by the lipids of the external half of the bilayer.

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