ENZYMATIC AND ELECTROPHYSIOLOGICAL CHANGES OF THE FUNCTION OF MEMBRANE PROTEINS BY CHOLESTEROL*

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Summary: Cholesterol accumulates in mammalian biological membranes suspended in aqueous solutions containing appropriate salts and buffers. Relatively small increases of the "normal"cholesterol content of mammalian membranes of different origin result in profound changes of the function of membrane proteins. Examples of enzymatic and electrophysiological changes are given.

Cholesterol is a normal constituent of biological membranes (1,2). It has been shown that on lipid bilayers (3) it exerts either a condensing or a liquifying effect, depending on whether the bilayer is, at the onset, in the liquid crystalline state or the crystalline state *per se*. [references in (4)]. Our long standing interest on the function of membrane proteins (5-8) was stimulated by our more recent observations (9), that cholesterol inhibits the binding of 5-OH-indole-acetaldehyde in brain homogenates. Here we report continuous changes in the activity and function of membrane proteins produced by loading of biological membranes with cholesterol.

Materials and Methods:

White male rats, 2 to 3 months old, and mongrel dogs (puppies) were used throughout. [4- ^{14}C]cholesterol (sp. radioactivity 57 Ci per mole) was purchased from The Radiochemical Centre, Amersham, England, and was brought to the desired sp. radioactivity by dilution with cholesterol. All reagents used in this study were of the highest commercial purity available.

Cholesterol Solutions (100% R.S**) were prepared by adding excess cholesterol into water, heating the mixture in a water bath, at 100°C, for 30 min under

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** Abbreviations: R.S. = relatively saturated cholesterol solutions in water; reported value about 2mg per liter (22). SPM=synaptosomal plasma membranes. ATP-ase =adenosine triphosphatase. NAD$^+$-ase=nicotinamide-adenine-dinucleotide pyrophosphate glycohydrolase. cpm = counts per minute.

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continuous stirring, while maintaining constant volume. After cooling (25°C), the mixture was filtered through an S@S #589, Red Ribbon filter paper. The 100% R.S. solution was usually 3.28×10^{-6}M (1.27 mg per liter), corresponding to 3.23×10^{-6} cpm. Its concentration was measured from the (known) sp. radioactivity of |4- ^{14}C|cholesterol. Appropriate salts and buffers were then dissolved in the cholesterol solutions, as required, for the various experiments (see below). Dilutions were prepared by mixing the 100% R.S. with cholesterol-free solutions of the same content in salts, etc.

Radioactivity was counted in 0.05 ml aliquots, with 1 ml Soluene and 10 ml scintillation fluid, containing, per liter toluene, 5g PPO and 0.3g POPOP, in a Packard Model 3320 β-counter, preset for 20,000 counts, with 80% efficiency.

Synaptosomal plasma membranes (SPM) (8), and homogenates freed from nuclei and debris (post-nuclear homogenates) (10) from brain or heart were prepared as described previously.

In enzymatic studies, cholesterol uptake by membranes was accomplished by mixing 0.4 ml aliquots of SPM or homogenate suspensions, containing 5 to 10 mg proteins (11), with 32 ml of appropriate cholesterol solutions. These mixtures were placed in 35 ml plastic centrifuge tubes and stirred at 25°C for 15 min. An identical control (cholesterol omitted) was prepared for background measurements. The completness of cholesterol uptake during treatment was checked in two ways: (a) To 5 ml aliquots of the mixtures CCl_3COOH solution was added to a final concentration of 5%. After centrifugation, the CCl_3COOH precipitates were dissolved in 5 ml Soluene, and 0.05 ml of the solutions were tranferred in counting vials; one ml Soluene, and 10 ml scintillation fluid were added and the samples were counted. To 4 ml aliquots of the acid supernatants, were added 1.2 ml of 1N NaOH. The mixture was extracted with 1 ml benzene, and 0.1 ml samples of the extract were used for counting. This procedure was followed in order to avoid, as much as possible, the quenching effect of CCl_3COOH in counting radioactivity. Similar acidification of |4- ^{14}C|cholesterol solutions with CCl_3COOH, in the absence of membranes or homogenates, and subsequent ultracentrifugation at 100,000 xg, did not alter the cholesterol content. (b) Alternatively, ultracentrifugation of 11 ml aliquots of the stirred suspensions of membranes at 100,000 xg for 60 min, was followed by resuspension of the pellets in cholesterol-free solution to an appropriate volume, and the radioactivities were measured in both the suspended pellet and the supernatant.

ATP-ase activity was measured essentially as described by Dunham and Glynn (12). Homogenates and SPM suspensions were prepared in TrisHCl, 0,32 Osm, pH 7.2 For preincubations, TrisHCl (20,86 g) and Tris (0,314 g) were dissolved per liter of 100% R.S. cholesterol solution, to give isotonic (0,32 Osm) solutions of pH 7.2. Incubations of one ml aliquots of the preincubated suspensions, for 60 min, at 38°C, with shaking, were performed in media containing 1.67 mM ATP, 16 mM NaCl, 16 mM KCl, 3 mM $MgCl_2$, in a total volume of 1.5 ml (12) Three controls with the enzyme omitted, containing the buffer and 1.0, 0.75 and 0.5 μmoles P_i respectively, and four additional controls with cholesterol omitted, containing 1.25, 0.75, and 0.5 ml of the suspension, accompanied all runs. EDTA (6 mM) was used in separate controls. P_i determinations were by the method of Fiske and Subbarow (13). Results are expressed either as P_i liberated per mg protein (11), referred to the degree of relative saturation with cholesterol of the preincubation media (homogenates), or to actual loading of the membranes with cholesterol (specific overload = moles cholesterol per mg of protein). The latter method of expression of the results was used when SPM and |4- ^{14}C|cholesterol were used. The approximate concentration of cholesterol in experiments with homogenates was 3.28×10^{-6}M, or 1.27 mg per liter (100% R.S.).

Preincubation of homogenates and of SPM, for studies of the NAD^+-ase activity, were identical with those described for ATP-ase. Incubations (14) for 15 min, at 38°C, in 1 ml total volume, with isotonic (0,2M) TrisHCl, pH 8.2, NAD^+ (6.42×10^{-4}M) and 0,5 ml of the suspensions of preincubated homogenates and SPM |0,524 and 0,366 mg of protein (11), respectively|. Hydrolyzed NAD^+ was determined, after deproteinization with 5% (final) CCl_3COOH, by measurements of the absorbance at 3270 Å, in the presence of approx. 2M NaCN (see Table I).

In electrophysiological experiments, we used Purkinje heart fibers from dogs. Methods of dissecting and recording intracellularly (KCl-filled micropipettes) have been described previously (15). Irrigation of the preparation (approx. 50 mg wet weight) with oxygenated normal or cholesterol-containing Tyrode solution (relative saturation with cholesterol, 100% R.S., approx. 3.25×10^{-6}M) was at a constant rate of flow, about 4 ml per min, at 36.5°C.

Results;

Cholesterol within the range used in these studies is taken up almost completely by SPM. Thus, in equilibrium experiments, quantities up to at least 1.15×10^{-7}moles of cholesterol, in a volume of 32.4 ml, were taken up by SPM, corresponding to 5-10 mg of proteins, simply by stirring the suspension at 25°C, for 15 min. Insignificant quantities of cholesterol remained in solution after deproteinization with CCl_3COOH and almost all the radioactivity is recovered in the precipitate. In the absence of acidification, only a small fraction of the cholesterol initially present in solution is recovered in supernatants from ultracentrifugation. Accumulation of appreciable cholesterol quantities in irrigation experiments is only a matter of time and rate of flow.

After it was established that both cholesterol uptake (see above) and the ensuing enzymatic changes (see below) occur in SPM, homogenates freed from debris and nuclei were used in routine enzymatic studies, instead of SPM, for convenience of preparation. The distribution of labeled cholesterol and the enzymatic changes were shown to be similar in SPM and post-nuclear homogenates.

The activity of the membrane exoenzyme (5, 16) NAD^+-ase decreases with increasing cholesterol load of the membranes (Table I). The loss of activity of this enzyme was used routinely to check the effectiveness and degree of overload of membranes with cholesterol.

Most striking results are obtained in electrophysiological studies. Intracellular recordings of spontaneously generated action potentials from a Pur-

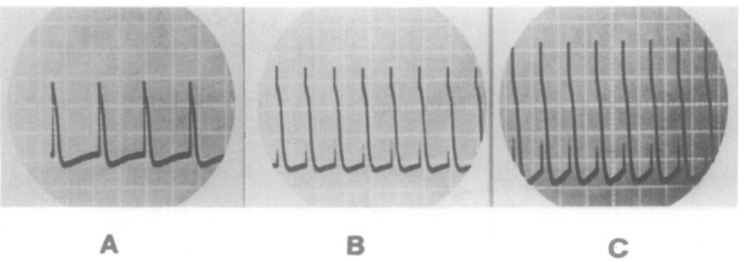

Fig. 1: Intracellular recordings of spontaneously generated action potentials from a Purkinje fiber of a young dog heart. Methology, as in (15). A: Normal activity. B and C : After 5 and 60 min of irrigation with oxygenated Tyrode saturated with cholesterol (3.28×10^{-6}M). Constant temperature, 36.5°C, Rate of irrigation, 4 ml per min, Amplification, 20 mV per vertical division, Time, 2 sec per horizontal division.

kinje fiber of young dog heart (see Methods and Figure 1) show hyperpolarization (from -80 to -100 mV), marked changes in the prepotential, increases in the amplitude of action potential and in the frequency of the spontaneously generated action potentials (up to 100% or more in individual experiments). The depolarizing current of the action potentials is tetrodotoxin insensitive (data not shown). Similar results were obtained in 12 different experiments.

These electrophysiological findings prompted us to investigate changes of the ATP-ase activity, both in the heart and the CNS. It was found to rise with increasing load of the membranes with cholesterol (Table I). The electrophysiological changes and the increased ATP-ase activity may occur independently, but increased frequency of augmented action potentials would be impossible in the absence of the supporting action of the pump.

Discussion:

The described changes of enzymatic activities are linear and continuous with increasing cholesterol load of the membranes and, whithin the range of cholesterol loading reported here, they may be expressed by the equation:

$$y = A \pm kx \qquad (1)$$

where y is the specific activity of a given membrane protein [e.g., an inte-

Table I. Adenosine Triphosphatase (ATP-ase) and Nicotinamide-Adenine-Dinucleotide Pyrophosphate Glycohydrolase (NAD^+-ase) Activities of Rat and Dog Homogenates (Heart and Brain) and of Dog Brain Synaptosomal Plasma Membranes (SPM), loaded with increasing quantities of [4 -^{14}C] cholesterol*.

Cholesterol	Homogenates				Cholesterol		Dog Brain Synaptosomal Plasma Membranes	
	ATP-ase			NAD^+-ase				
% R.S.	Rat Heart	Dog Heart	Rat Brain	Rat Brain	% R.S.	Moles x 10^{-8} per mg protein	ATP-ase	NAD^+-ase
0	1.52	0.57	1.23	16.34	0	0.00	1.17	16.10
					20	1.79	1.37	12.99
25	1.70	0.63	1.41	12.32				
					40	3.05	1.56	9.89
50	1.87	0.69	1.57	8.23	50	4.09	1.67	8.47
					60	4.86	1.78	8.19
75	2.12	0.77	1.74	6.48				
					80	6.44	2.02	3.67
100	2.28	0.83	1.92	2.38	100	8.32	2.10	0.85

*Results are expressed as P_i liberated per 60 min (ATP-ase) and NAD^+-ase hydrolysed per 15 min (NAD^+-ase) both per mg protein (11), referred to the degree of relative saturation with cholesterol of the preincubation media (homogenates), or to actual loading of the membranes with cholesterol (specific overload = moles cholesterol per mg protein). The latter method of expression of the results was used when SPM and [4 -^{14}C]cholesterol were used. The approximate concentration of cholesterol, in experiments with homogenates, was 3.28 x 10^{-6}M, or 1.27 mg per liter (100% R.S.). P_i-liberated in moles x 10^{-6}. NAD^+ -hydrolysed in moles x 10^{-7}.

gral (membrane) enzyme*] and A is a value representing the specific activity of a membrane protein, at the level of the "physiological" cholesterol content of a particular mammalian membrane, i.e., before extra additions or losses. The variable x denotes the change of cholesterol content of the membrane, and the constant k (slope of the line) corresponds to the susceptibility of a given membrane protein to the effect of cholesterol. We assume that cholesterol enters inbetween the phospholipids of the bilayer (17), thus increasing the "normal" cholesterol : phospholipid ratio for a given biological membrane.

It should be noted that discontinuous changes of enzymatic activities reported previously are due to phospholipid replacements (18) or reconstitutions of the so-called "annulus" (19), i.e., the immediate mantle of phospholipids surrounding and shaping membrane proteins. Such changes may be achieved by more drastic means, compared to those reported here, i.e., use of detergents, sonication, etc.

It is noteworthy, that the overload of cholesterol required for the appearance of signs of disfunction of a membrane is quite small, in comparison to the normal cholesterol content of a membrane (1, 2). These phenomena, then, appear to be similar to those described by Sheetz and Singer (20), with the difference that cholesterol is uncharged and can approach the membrane from either side, with its polar hydroxyl group orientated towards the surrounding polar medium.

It is tempting to speculate that any disturbance of the subtle equilibrium between the cholesterol which is carried in the plasma (approx. 240 mg%) mostly by low density lipoproteins (21), and the carriers themselves, may lead to escape of traces of free cholesterol into the aqueous phase. It would be of interest to search for such traces of free cholesterol in plasma.

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* In more recent experiments, we found that the rise of functionality of the electrophysiological phenomena is also a linear function of the (continuously) rising load of the membrane with cholesterol, as effected during irrigation.

(S.G.A.A. and C.P.) carried out some initial studies on the effect of cholesterol on serotonergic receptors (now in preparation). The preliminary results of that work, at a time (summer of 1976) when our laboratory was not fully equipped, encouraged us to pursue, in Athens, studies reported here, on the effects of cholesterol on biological membranes.

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