Temperature effect of cholesterol association with synaptosomal plasma membranes of rabbit brain

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(Received 1 June 1984/Accepted 17 July 1984)

Association of exogenous cholesterol with rabbit brain synaptosomal plasma membranes follows an exponential path described by the general formula $y = a \times e^{bx}$. The co-operative nature of this association was shown when increasing amounts of unlabelled cholesterol glucoside (up to 0.5 mM) were added to a fixed amount (5 μ M) of [14 C]cholesterol, when a biphasic curve of the binding of [14 C]cholesterol into the membranes was obtained. Arrhenius plots of this association revealed two break points which occur at 25°C and 42°C. The first break apparently corresponds to the transition from the crystalline to the gel phase. The second break may be due to the (continuously) increasing entropy of the system which creates at a certain point difficulties in the binding of cholesterol into the lipid bilayer.

In previous communications we reported on the ability of monomeric cholesterol in aqueous solutions to produce functional changes of integral proteins of biological membranes. These changes depend on the concentration of cholesterol in solution, the quantities of membranes present and the time of exposure of the membranes to it (Alivisatos *et al.*, 1981*a,b*; Delicostantinios, 1983). The water-soluble glucoside of cholesterol was reported to show identical binding and evoked effects on biomembranes similar to those of cholesterol (Alivisatos & Delicostantinios, 1982). The introduction of this compound in our studies permitted a better understanding of the nature of cholesterol association with biomembranes. Although different methods for the study of the kinetics of cholesterol association with biomembranes have been developed, using for example cholesterol dissolved in detergents (Razin *et al.*, 1974) or plasma lipoproteins (Gottlieb, 1980) or liposomes containing cholesterol (Lange *et al.*, 1979), the methodology involving 'aqueous solutions' of cholesterol present in our study has the advantages of the easy application of relatively simple mathematical formulations.

A study of the kinetics of cholesterol association with the synaptosomal plasma membranes of rabbit brain and the effect of temperature on this association is reported here.

Materials and methods

Synaptosomal plasma membranes from rabbit brain were prepared and qualitatively assessed as

previously described (Papaphilis & Delicostantinios, 1980), from Ficoll-purified and then osmotically soaked synaptosomes, by ascending fractionation through a discontinuous sucrose density gradient. Compared with the original homogenate from which they were prepared, they showed a 7–10-fold increase in the specific activity of the ouabain-sensitive ATPase (EC 3.6.1.3).

Synaptosomal plasma membranes were extracted overnight with 20 vol. of chloroform/methanol (2:1, v/v) according to the method of Folch *et al.* (1957). Phospholipids were separated from the chloroform extracts by t.l.c. on silica gel G with appropriate standards, with a solvent system of chloroform/methanol/acetic acid/water (25:15:4:2, by vol.). The phospholipids were located with iodine vapour, removed and the content of lipid phosphorus was measured in the spots by the method of Bartlett (1959) after digestion with 70% HClO₄ at 180°C. More than 95% of the phospholipid phosphorus chromatographed with the standard phospholipid. Cholesterol was determined by g.l.c. with 5 α -cholestane as an internal standard. Protein content was determined by the Lowry *et al.* (1951) method with bovine serum albumin (Sigma) as standard.

Cholesterol (Merck AG) was routinely recrystallized twice from 95% ethanol and its purity was tested by g.l.c. Cholesterol glucoside was synthesized as previously described (Alivisatos *et al.*, 1981*a*). Aqueous 'solutions' of cholesterol or its glucoside were prepared by refluxing the appropriate quantities under O₂-free N₂ for 1 h, with continuous stirring with a magnetic bar.

Preincubations of synaptosomal plasma membranes (0.15mg/ml) with the different concentrations of [^{14}C]cholesterol specified in Fig. 1 were for 3h at 35°C in a final volume of 5ml. The mixtures were buffered with 5mM-Tris/HCl (pH 7.4)/0.15M-NaCl and were stirred magnetically. Preincubations of the membranes with the different amounts of unlabelled cholesterol glucoside specified in Fig. 2 were always carried out for 3h at 35°C in an incubation mixture of 5mM-Tris/HCl (pH 7.4)/0.15M-NaCl containing 0.15mg of protein/ml in a final volume of 5ml under continuous magnetic stirring. Preincubations of ^{14}C -labelled cholesterol ($5 \times 10^{-6}\text{M}$) with 6mg of membrane protein were also performed in a 40ml final volume of 5mM-Tris/HCl (pH 7.4)/0.15M-NaCl. They were stirred magnetically at the specified temperatures (Fig. 3) and aliquots (4ml) were withdrawn at different time intervals. They were layered over 30ml of 20% (w/v) sucrose/5mM-Tris/HCl, pH 7.4, and centrifuged for 1h at 95000g in the SW-27 rotor of a Beckman L5-75 ultracentrifuge. Synaptosomal plasma membranes used for measurement of [^{14}C]cholesterol binding were dissolved in 1ml of 1% sodium dodecyl sulphate and counted in 10ml of a dioxan-based scintillant (Bray's solution). C.p.m. were converted to nmol by use of the known specific radioactivity. The constant amount of radioactivity which was absorbed to membranes at zero time (instantaneous deposition of radioactivity) was subtracted in every case from all samples. Cholesterol binding is expressed as nmol of cholesterol/mg of membrane protein.

Following incubation of synaptosomal plasma membranes with cholesterol, the bound and free [^{14}C]cholesterol fractions were separated by centrifugation as described above and then pooled separately. They were extracted by the method of Folch *et al.* (1957) and then subjected to g.l.c. as mentioned above. The extracts of membrane-bound and free [^{14}C]cholesterol moved identically with those of unincubated [^{14}C]cholesterol, suggesting that no significant metabolic conversion of membrane-bound or free [^{14}C]cholesterol occurred under the incubation conditions used.

Results and discussion

Cholesterol association with synaptosomal plasma membranes, within the limits of cholesterol concentrations (up to 5 μM) achieved with this hydrophobic compound, follows an exponential curve of the general formula $y = a \times e^{bx}$ (Fig. 1). In this expression y represents the amount of bound [^{14}C]cholesterol/mg of membrane protein, x is the concentration of [^{14}C]cholesterol in the incubation medium, and a , the zero-time intercept, and b , in the exponent, are constants. Under the conditions

Fig. 1. Binding of [^{14}C]cholesterol into synaptosomal plasma membranes

Experimental details are given in the text. Computer analysis for curve fitting was performed with a programmable Hewlett-Packard HP-97 calculator. The correlation coefficients (r^2) for exponential fitting were above 0.95. The indicated values represent average estimates of duplicate determinations of a typical experiment, which has been repeated several times.

of incubation used (see the Materials and methods section) and due to the limitations inherent in the insolubility of cholesterol in aqueous media (only up to 5 μM), saturation of the binding could not be achieved. That there may exist a co-operativity in cholesterol association with synaptosomal plasma membranes is, however, not clearly established (imperfect Hill plot of binding) due to limitations mentioned (inaccessibility of saturation levels) in this type of experimentation.

Introduction of cholesterol glucoside, to overcome the limitations in the insolubility of cholesterol, allowed a demonstration of the co-operative nature of this association process. Thus, as shown in Fig. 2, when increasing amounts of unlabelled cholesterol glucoside (up to 0.5mM) were added to a fixed amount of (5 μM) of ^{14}C -labelled cholesterol, a biphasic curve of the binding of [^{14}C]cholesterol into synaptosomal plasma membranes was obtained. Up to 0.1 mM-cholesterol glucoside, there was increased [^{14}C]cholesterol binding; above this concentration of cholesterol glucoside progressive displacement was observed. At 0.5mM-cholesterol glucoside, 90% of the radioactivity was displaced.

Fig. 2. Successive stages of ^{14}C -labelled cholesterol binding into synaptosomal plasma membranes in the presence of changing concentrations of cholesterol glucoside

At a concentration of cholesterol (constant) of $5\ \mu\text{M}$, its binding in the absence of glucoside is approx. 4.2 nmol/mg of membrane protein. In the presence of glucoside, positive co-operativity phenomena may occur, increasing appreciably the binding of ^{14}C cholesterol ($\sim 5.5\text{ nmol/mg}$ of protein). At higher concentrations of the glucoside mutual exclusion with cholesterol prevails. Points and bars represent means and S.D. respectively, for three different determinations.

in terms of molarities. To overcome this problem, we were obliged to determine the quantities of cholesterol ($550 \pm 40\text{ nmol}$) and phospholipids ($680 \pm 60\text{ nmol}$) per mg of protein in synaptosomal plasma membranes. The difference of these two quantities (130 nmol/mg of protein) was taken as the maximal possible capacity of membranes that could associate with cholesterol. It was implicitly assumed that a cholesterol/phospholipid ratio of 1:1 is the maximal allowable ratio for these membranes. Although this simplification is not strictly correct it is sufficient to serve our purposes for this instance.

The integral form of the second order rate law is:

$$k_f t = \frac{1}{a-b} \ln \frac{b(a-x)}{a(b-x)}$$

where a is the initial concentration of the maximal membranous capacity ($19.5 \times 10^{-6}\text{ M}$), b is the initial concentration of cholesterol present in the incubation mixture ($5 \times 10^{-6}\text{ M}$) and x is the cholesterol associated/mg of membrane protein at time t .

When $\ln[b(a-x)/a(b-x)]$ was plotted against time, a straight line was observed and linear regression analysis was used for the estimation of the correlation coefficient ($r^2 = 0.99$) and of the slope.

The forward rate constant, k_f , changes with changing temperature. The dependence of k_f on temperature is a reflection of activation energy as expressed by the Arrhenius equation

$$\ln k_f = \frac{-E_a}{R} \frac{1}{T} + \text{constant}$$

where E_a is the activation energy, R the gas constant ($8.314\text{ J}\cdot\text{K}^{-1}\cdot\text{mol}^{-1}$) and T the absolute temperature. The value of the activation energy E_a may be obtained from the plot of $\ln k_f$ against $1/T$. The slope of the line is $-E_a/R$, from which E_a can be evaluated.

As indicated in Fig. 3, an Arrhenius plot of the natural logarithms of k_f versus the absolute temperature shows two break points. One of these occurs at lower temperatures (around 25°C) and corresponds to the melting profile of the membrane, i.e. a lipid phase separation (Demel & DeKruyff, 1976; Tsakiris & Deliconstantinos, 1984). The second break occurs at 42°C . At low temperatures the association of cholesterol with the membranes is severely limited. Thus, from 22°C to 25°C the activation energy (E_a) reaches a value of 569 kJ/mol ; within the range $25\text{--}40^\circ\text{C}$, only 14.6 kJ/mol required for the 'activation'. Thus, when the incubation temperature was increased from low temperatures ($<25^\circ\text{C}$) to temperatures higher than 25°C a drop in energy of activation (E_a) was observed. The drop in E_a

The biphasic nature of this curve may suggest positive co-operativity in the binding of cholesterol into synaptosomal plasma membranes, with at least one class of high-affinity specific binding sites (Deliconstantinos & Ramantanis, 1983; Akera & Cheng, 1977).

Cholesterol association with the membranes depends both on the concentration of the reactants and time. At various quantities of cholesterol and membrane protein, the association of cholesterol with membranes followed second-order kinetics, as deduced from various tests of equation fitting of the experimental data. A plot of cholesterol association with synaptosomal plasma membranes versus time is linear for at least the first 4 h, with correlation coefficients of the order of >0.98 and deviations less than 2%. Second-order kinetics requires the membrane quantities to be expressed

Fig. 3. Arrhenius plot of the natural logarithms of the second order rate constants (k_t) of cholesterol binding into synaptosomal plasma membranes, versus the absolute temperature

The drawn curves represent a typical experiment which has been repeated three times and are linear regressions through points which are mean values of duplicate estimates ($r^2 > 0.98$).

occurring when the temperature is raised is connected with a decrease in entropy of activation (ΔS):

$$\Delta S_1 - \Delta S_2 = \frac{E_{a1} - E_{a2}}{T_0}$$

where E_{a1} and E_{a2} indicate the activation energies of the binding at temperatures below and above the break, respectively (Rene *et al.*, 1978). Considering the state of the membrane, this means that raising the temperature above the 25°C break-point coincides with the appearance of an ordered process during the binding. Around the second break point (42°C) the value of E_a becomes suddenly negative, i.e. despite a higher kinetic energy of the molecules of cholesterol, their association with the membranes is limited. This limitation of association is not due to coagulation phenomena, which would occur at much higher temperatures, (above 60°C). Thus, between 42 and 44°C the activation energy becomes -79.5 kJ/mol, and it continues to drop at higher temperatures. Thermodynamically, this is, most probably, due to temperature-dependent conformational changes of

the membrane bilayer (Kimelberg & Papahadjopoulos, 1974). This fits in with the idea that at sufficiently high temperatures the disorder that prevails within the structure of the bilayer may prevent the association of cholesterol molecules with the membranes.

In conclusion, Arrhenius plots of the binding of cholesterol into synaptosomal plasma membranes exhibited three slopes with two breaks. The first break (25°C) apparently corresponds to the transition from the crystalline to the gel phase. The second break (42°C) may be due to the (continuously) increasing entropy of the system which creates at a certain point difficulties in the binding of cholesterol into the bilayer.

I am indebted to Professor S. G. A. Alivisatos for the valuable criticism and help he provided during the preparation of this manuscript. This work was supported by a grant from the University of Athens (22491/8797).

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