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COOPERATIVE NATURE OF THE BINDING OF CHOLESTEROL ON TO SYNAPTOSOMAL PLASMA MEMBRANES OF DOG BRAIN *

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Summary

The 'binding' of cholesterol on to dog brain synaptosomal plasma membranes from aqueous cholesterol 'solutions' was studied. 'Binding' of exogenous cholesterol is a slow process, strictly depending on the concentration of cholesterol and the quantity of the membranes present. It appears that binding probably occurs in three distinct successive stages. The first stage occurs very rapidly, and consists of a large deposition-like accumulation of cholesterol onto the membranes. This stage is characterized by the lack of functional changes of integral proteins. It is followed or accompanied by a slower type of binding, probably at 'specific binding sites', the nature of which is, in all probability, cooperative. Thus, when the glucoside of cholesterol is used at lower concentrations as compared to cholesterol it increases the binding of cholesterol, while at higher concentrations relative to cholesterol, it antagonizes its binding. This stage, which evokes strong functional changes of integral proteins, merges without interruption into an incorporation of cholesterol as a structural element into the membranous framework (nonspecific binding).

The common source of problems arising in attempts to study the kinetics of binding of the exogenous cholesterol onto biomembranes is the hydrophobic

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nature of the compound. Cholesterol is said to be soluble in water at concentrations up to 2 mg/l (approx. 5 μM) [1]. This was reinvestigated [2] with approximately the same results (approx. 1.8 mg/l) and a thermodynamically reversible critical micellar concentration (25—40 nM at 25°C) was established.

Although many methods of changing the cholesterol content of biomembranes have developed, i.e., with liposomes [3,4] and with lipoproteins [5], etc., the methodology involving 'aqueous solutions' of cholesterol [6] has many advantages. It is, for instance, the fastest for a meaningful enrichment of a membrane with cholesterol. It also allows the use of simple mathematical formulations, provided that a homogeneous distribution of mono- or polymeric cholesterol particles (i.e., single shaped and single sized) would collide with the membrane particles. The good fit of experimental points with curves derived in this manner, would provide full justification for such an empirical procedure. We have, in the past, observed functional changes of integral proteins evoked by incubation of cholesterol 'aqueous solutions' with synaptosomal plasma membranes [6—8]. 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. They extend, in a continuous fashion, over large areas of changing concentrations and times of exposure and they are obviously related to these variables by simple physicochemical laws.

Cholesterol (Merck A.G.) was routinely recrystallized twice from 95% ethanol. At the beginning of this study, the purity of the steroid was tested on 0.25 mm thick commercial silica gel precoated TLC plates (Merck). Chromatography was developed with *n*-heptane/ethyl acetate (1 : 1, v/v) [9]. The band containing cholesterol was scraped off and the steroid was eluted with a chloroform/methanol 4 : 1, v/v) mixture. The eluate was then evaporated under nitrogen (free of oxygen) and cholesterol, redissolved in 95% ethanol was stored at —20°C until use. Similar purification and storage was also applied to appropriately isotopically diluted [4-^{14}C]cholesterol (specific radioactivity 57.8 Ci/mol, Radiochemical Centre, Amersham, U.K.). During the studies described here, it was established that the binding of non-purified cholesterol onto membranes evokes exactly the same functional changes as cholesterol purified as described above. It was also found that the rate of the binding of recrystallized cholesterol onto membranes was not appreciably altered by further purification [10]. Besides, the binding quantities, minimal as they were, were far larger than any impurities possibly present in the recrystallized preparations.

Aqueous 'solutions' of cholesterol were prepared as previously [6] by refluxing the appropriate quantity of cholesterol under nitrogen (free of oxygen) atmosphere for 1 h under continuous stirring with a magnetic bar. No detectable decomposition of thus treated cholesterol could be found by the thin-layer chromatography method mentioned above.

Dog synaptosomal plasma membranes were prepared as previously [8]. Checked under the electron microscope, these preparations were found free of nuclei and mitochondria. They consisted mainly of membranous fragments and a few vesicles. They were further tested enzymatically. Compared to the original homogenate from which they were prepared, various batches showed 7- to 10-fold increase of their ouabain-sensitive ATPase (EC 3.6.1.3) [11] and the NAD^+ nucleosidase (EC 3.2.2.5) [12] activities. Batches of membranes were

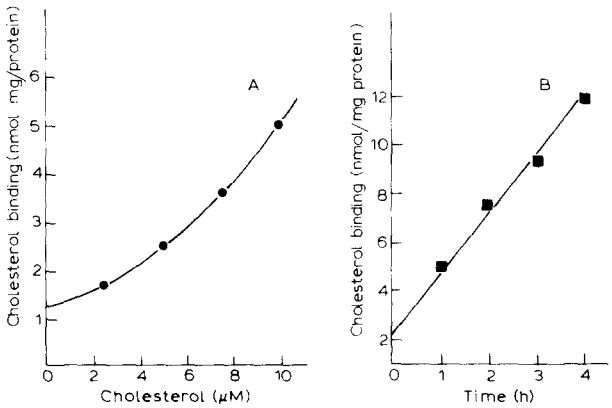

Fig. 1. A. Binding of [^{14}C]cholesterol on synaptosomal plasma membranes. [^{14}C]Cholesterol at concentrations shown in the absissa, was incubated with 0.124 mg protein/ml for 1 h at 37°C. B. Binding of [^{14}C]cholesterol on synaptosomal plasma membranes as a function of time. The [^{14}C]cholesterol concentration was 10 μM; synaptosomal plasma membranes proteins were 0.124 mg/ml or 16.8 μM 'binding sites'. Other experimental details are given in the text. The results are regressed in (A) exponential fashion ($y = a\ \exp(bx)$, $a = 1.229 \cdot 10^{-9}$, $b = 0.142$) ($r^2 = 0.995$) and (B) in a linear fashion ($r^2 = 0.996$). Regression of the same points (B) in a power fashion (without intercept) gave $r^2 = 0.994$. The indicated values represent average estimates of duplicate determinations of a typical experiment which has been repeated six times.

prepared usually from 30 g of young dog brain, or multiples of it. 1 g wet tissue correspond to approx. 4 mg synaptosomal plasma membrane protein. The membrane lipids from an aliquot of each batch were extracted [13] and the content in cholesterol [14] and phospholipids [15] were measured.

Incubations of appropriate quantities of membranes with cholesterol were usually performed in 10 to 20 ml final volume at the concentrations of cholesterol specified in the various figures. The mixtures were 5 mM with respect to Tris-HCl (pH 7.4) and 0.15 M with respect to NaCl. They were stirred magnetically at 37°C and aliquots (2—4 ml) were withdrawn at specified time intervals (see figures). These aliquots were layered over 30 ml of 20% sucrose/5 mM Tris-HCl (pH 7.4), and centrifuged for 1 h at 95 000 × *g* in the SW-27 rotor of a Spinco L5-75 ultracentrifuge. The precipitate of this centrifugation, which consisted of the membranous material largely freed of adhering cholesterol, was suspended in 1 ml of 5 mM Tris-HCl (pH 7.4). Samples were used for protein [16] and radioactivity determinations. The latter was determined by dissolution of the membranes with 0.1 ml 10% sodium dodecyl sulfate and mixing with 10 ml dioxane-based scintillation fluid [17]. Efficiency of counting was estimated, when necessary, by the use of internal standards. Determination of the ouabain-sensitive ATPase activity was used occasionally as a functional parameter, indicating the binding of cholesterol onto the synaptosomal plasma membranes.

The initial (1 h) binding of different [^{14}C]cholesterol concentrations in or on to synaptomal plasma membranes is shown in Fig. 1A. In such experiments, the

original quantity of 'binding sites' * in the incubation mixture (16.8 μM) is higher in comparison to the exogenous cholesterol concentration shown in the absissa. The experimental values follow closely an exponential curve of the general formula $y = ae^{bx}$. In this expression y represents the amount of bound cholesterol per mg synaptosomal plasma membranes protein and x is the concentration of cholesterol in the incubation medium; a, the zero-time intercept and b in the exponent, are constants. This experiment was repeated several times. When synaptosomal plasma membranes of the same batch was used for the repetitions, the results were very close to each other. With synaptosomal plasma membranes from different batches, the results were qualitatively the same, but there could be differences in the constants (a and b).

Since cholesterol binding depends both on the concentration of the reactants and time, attempts were made to apply kinetics of either first or second order. In Fig. 1B, [^{14}C]cholesterol binding was measured at times indicated in the absissa. On this basis we used (Fig. 2A) Eqn. 1:

$$\ln \frac{a}{a-x} = kt \tag{1}$$

for determination of a pseudo-first-order constant k; where a corresponds to the initial concentration of exogenous (labeled) cholesterol and x to its fraction bound at a given time t.

$$\frac{1}{a-b} \ln \frac{b(a-x)}{a(b-x)} = kt \tag{2}$$

was used for the determination of a (pseudo)second-order constant k (Fig. 2B); where a is the quantity of 'binding sites', b is the concentration of cholesterol present in the incubation medium and x and t have the same meaning as in Eqn. 1. At the quantity of synaptosomal plasma membranes and the concentration of cholesterol used in this experiment (Fig. 1B), the k values obtained were $6.334 \cdot 10^{-8}$ s^{-1} for a first-order and $3.775 \cdot 10^{-3}$ M$^{-1} \cdot$ s^{-1} for a second-order process (Fig. 2, A and B). The experimental points from which these determinations were made fell on a straight line, the determination coefficient of which was $r^2 = 0.996$, indicating minimal, if any, deviation of the experimental points from the regression line (Fig. 1B). A justification for the introduction of a in Eqn. 2 comes from the results in Table I, where 'isomolar' concentrations of cholesterol and 'binding sites' were used. The binding of cholesterol per mg synaptosomal plasma membranes protein was the same, even if the initial quantities changed by approx. 3-fold.

The results in Fig. 2 (plots A and B) indicate, that both first- and second-

* The number of cholesterol 'binding sites' on the synaptosomal plasma membranes was provisionely and arbitrarily estimated here to be 130 ± 20 nmol/mg protein. This value was assessed from the differences between the indigenous cholesterol (C) and phospholipid (P) levels in synaptosomal plasma membranes, which, in five different preparations of synaptosomal plasma membranes gave figures 550 ± 40 nmol/mg protein and 680 ± 60 nmol/mg protein, respectively (C/P ≈ 0.82). It was implicitly assumed that a C/P ratio of 1 : 1 is the maximal tolerable ratio for the synaptosomal plasma membranes. Occasional reports in the literature point towards the possibility of higher (cf., Refs. 18 and 19) than the 1 : 1 ratio used in our calculations. This, however, would only affect the numberical values of constants determined in this report (see Results).

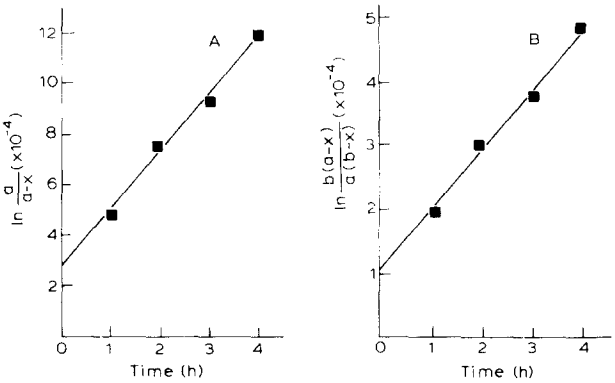

Fig. 2. A. Pseudo-first-order plot of cholesterol on synaptosomal plasma membranes; a is the initial concentration (10 μM); x is the cholesterol bound in nmol/mg protein at any time (t) shown in Fig. 1B. $k = 6.334 \cdot 10^{-8}\ s^{-1}$. B. (Pseudo)-second-order plot of cholesterol binding on synaptosomal plasma membranes; a is the concentration of 'binding sites' (16.8 μM); b is the initial concentration of cholesterol (10 μM); x is the cholesterol bound in nmol/mg protein at any time (t). $k = 3.775 \cdot 10^{-3}\ M^{-1} \cdot s^{-1}$.

order kinetics would, at a first appearance, serve the purpose of describing the binding of cholesterol on (in) to synaptosomal plasma membranes. It is evident, however, that k values change considerably and in an orderly manner. For example, this is the case in the experiments in Fig. 3. In this instance the cholesterol concentration was kept constant throughout, while the quantities of synaptosomal plasma membrane protein (e.g., the number of binding sites) change. It is evident that the binding of cholesterol per mg synaptosomal plasma membranes protein and the second-order k values, calculated as above (Fig. 2B) changed in a power fashion. The significant point here is that while the number of binding sites increases, cholesterol binding per mg synaptosomal membranes proteins decreases, despite the presence of an excess of exogenous cholesterol.

TABLE I

CHOLESTEROL BINDING ONTO SYNAPTOSOMAL PLASMA MEMBRANES FROM 'ISOMOLAR' CONCENTRATIONS OF [^{14}C]CHOLESTEROL AND MEMBRANE 'BINDING SITES' IN 3-h INCUBATION TIME

The indicated values represent average estimates of duplicate determinations of a typical experiment which has been repeated three times.

Cholesterol or membrane 'binding sites' concentration (specific radioactivity of cholesterol 6916 cpm/nmol (μM)	Actual input of membrane quantities (mg protein/ml)	Protein recovery after gradient centrifugation (mg protein/ml)	Radioactivity recovery after gradient centrifugation (cpm/ml)	Binding of cholesterol (nmol/mg protein)
3.75	1.387	0.050	2998	8.672
5.00	1.850	0.070	4313	8.910
6.25	2.343	0.090	5432	8.728
7.50	2.805	0.095	6062	9.227
8.75	3.237	0.120	7632	7.883
10.00	3.700	0.140	7817	8.074
			Mean value:	8.582 ± 0.51

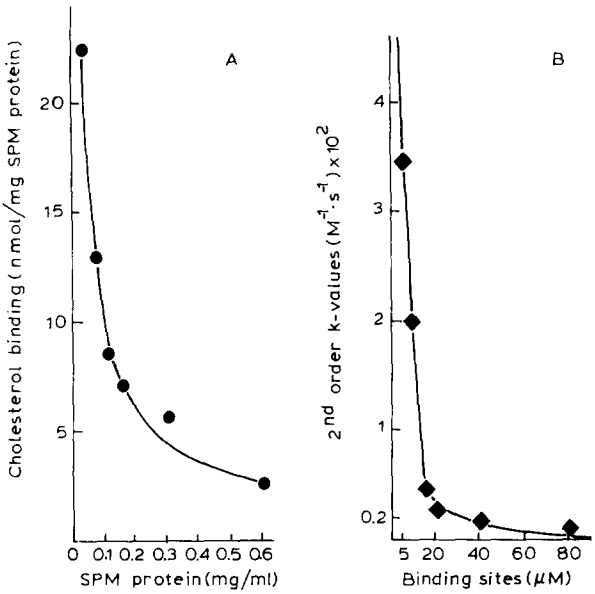

Fig. 3. Changes of the cholesterol binding on synaptosomal plasma membranes (SPM) (A) and of the second-order rate constant (k) values (B) with changing quantities of the SPM protein or their corresponding 'binding sites' shown in the absissa. Initial cholesterol concentration, 10 μM. Experimental details are given in the text. A. $y = ax^b$, $b = -0.755$, $r^2 = 0.97$. B. $y = ax^b$, $b = -1.66$, $r^2 = 0.99$.

These findings indicate that binding of cholesterol onto synaptosomal plasma membranes does not strictly obey first- or second-order kinetics, although it depends on the concentration of both cholesterol and the binding sites. The results in Fig. 3 prompted us to think of a cooperative type of interaction, in which, the k values, whether of first or second order, change with the quantities of cholesterol and the membranes in the experiment.

To test this, we performed experiments with using cholesterol glucoside. The glucoside is much more water soluble, as compared to cholesterol, and it evokes similar functional changes of integral proteins as cholesterol does (see following communication) [20]. This permitted an experiment in which unlabeled glucoside of cholesterol and labeled [^{14}C]cholesterol were incubated together with synaptosomal plasma membranes. In a time-course experiment of 3 h, when labeled [^{14}C]cholesterol (8 μM) plus unlabeled cholesterol glucoside (2.5 μM) were incubated with 0.35 mg synaptosomal plasma membrane protein per ml of incubation mixture, the glucoside increased the binding of [^{14}C]cholesterol by 32%, as compared to the [^{14}C]cholesterol alone. At higher concentrations of cholesterol glucoside (28 μM), however, there was a displacement of the labeled cholesterol, so that binding of [^{14}C]cholesterol dropped by 60%, as compared to the [^{14}C]cholesterol alone. This effect asserts the hypothesis of a 'positive cooperativity' in the binding of cholesterol onto synaptosomal plasma membranes.

Attempts, however, to apply the Hill equation, or perhaps more appropriately, use the Koshland-Nemethy-Filmer approach [21,22], were abandoned, since for such attempts the system must not only be reversible (see above) but also at equilibrium. This is almost impossible to achieve with cholesterol as the

ligand. The solubility of cholesterol in water is very low and the process of its uptake by membranes is so slow, as to make equilibrium studies under our conditions almost impossible.

In previous attempts to elucidate the kinetics of binding of exogenous cholesterol onto biomembranes, it has often been assumed that bound exogenous cholesterol does so by following Michaelis or Langmuir isotherms [23]. What is presented here is against these hypotheses, for the following reasons: first, the initial stages of binding do not seem to follow strictly first- or second-order kinetics; second, the displacement of [^{14}C]cholesterol by high concentrations of the cholesterol glucoside, and the enhanced binding of [^{14}C]cholesterol by lower concentrations of the glucoside lead to the conclusion that the binding has a 'positive cooperative' character (see above). In terms of a Monod-Wyman-Changeaux model [24] of the 'binding', it could be argued that 'specific sites' on the membrane may oscillate between T and R forms. In addition, our present results indicate that at the higher concentrations used in our studies (especially, in experiments with displacers; see following paper [20]), exogenous cholesterol might overcome the barrier imposed by the 'specific sites' and incorporate further, as a structural element, into the membrane framework. In such a case, only their extra cholesterol might follow the assumptions mentioned in the footnote on p. 645. The extra bound or 'incorporated' cholesterol could be regarded as 'nonspecifically bound' and would be responsible for the changes of the membrane fluidity already established [25].

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