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SHORT COMMUNICATIONS

Modulation of serotonergic receptors by exogenous cholesterol in the dog synaptosomal plasma membrane

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It has been reported previously that cholesterol from aqueous solutions is incorporated in the structure of biological membranes, producing deep functional changes of membrane-associated enzymes [1]. Changes in the activities of the enzymes (Na^+-K^+)-ATPase and NADase affected by cholesterol afford such examples at the synaptosomal plasma membrane level. Recently it has been shown that cholesterol modulates the activity of Ca^{2+}-dependent ATPase of the sarcoplasmic reticulum [2]. It has also been demonstrated that in the intact cells of the Purkinje heart fibers from young dogs, cholesterol, when included in the irrigating Tyrode solutions at a concentration of approx. 5×10^{-6} M (saturation level), increased markedly in the presence, but not in the absence, of Ca^{2+} the amplitude and the frequency of the spontaneously generated action potentials in those cells [3].

These data indicate that loading *in vitro* of biological membranes with cholesterol, beyond the indigenous membrane cholesterol levels, results in deviations from the normal physiological function of these membranes in a number of processes. In this communication we extend our study on the sensitivity of biological membranes to cholesterol and report on the effect of cholesterol on the activity of serotonergic receptors in the synaptosomal plasma membrane.

Synaptosomal membranes (SPM) from dog brain were prepared from ficoll-purified [4] and subsequently osmotically soaked synaptosomes by ascending fractionation through discontinuous sucrose density gradient [5]. The quality and the degree of purification of the isolated membranes (fraction at the interphase between 32% and 28.5% (w/w) sucrose) was assessed by electron microscopy (results not shown) and by following, through the steps of purification, the activities of succinic acid dehydrogenase (SDH, a mitochondrial marker [6]) and lactic acid dehydrogenase (LDH, a cytoplasmic marker). Only those fractions of SPM which showed a reduction by over 95 per cent of the activity of SDH of the mitochondrial fraction and of the activity of LDH of the fraction of synaptosomes were used in this study.

At incubation of synaptosomal membranes with saturated, aqueous [^{14}C]cholesterol which was buffered with 0.004 M Tris–HCl, pH 7.4, over a period of 3 hr at 35° with constant stirring, the steroid was incorporated in the membrane in amounts not exceeding 10 nmoles cholesterol per mg of protein. The separation of bound from non bound cholesterol was achieved by centrifuging the incubated membranes through a cushion of 20% sucrose, buffered with the same medium, for 60 min at 95,000 *g* (Beckman SW 27 rotor). This allowed us to study the kinetics of cholesterol binding by SPM, by way of obtaining incorporation curves with minimal background levels and with a statistical value for the correlation coefficients $r^2 > 0.98$.

Uptake of 5-hydroxytryptamine (5-HT, serotonin) by SPM was measured as follows: Membrane suspensions, containing 0.3 to 0.4 mg of protein per ml of Krebs–Ringer buffer (KRB, consisting of 20 mM Tris–HCl, 132 mM NaCl, 4.8 mM KCl, 2.4 mM $MgSO_4$ and 1.8% (w/v) glucose, adjusted to pH 7.4), were pre-incubated with 5×10^{-4} M harmine (an MAO inhibitor) for 15 min at 35°. Thereafter, withdrawn aliquots containing 0.15 mg of protein were incubated, to a total volume of 1 ml, with varying concentrations of [G-^{3}H]-5-HT (10–20 Ci/mmole, Radiochemical Centre, Amersham) in the presence or absence of 10 μM 5,6-dihydroxytryptamine (5,6-DHT). The incubation was run for 30 min at 35° and stopped by rapid filtration under suction through HAWP millipore filters (0.45 μ pore diameter) using a Millipore 3025 sampling manifold. Filters were washed twice with 5 ml ice-cold KRB and transferred into counting vials. They were then shaken in 0.8 ml of SDS, dissolved in 1 ml of 2-methoxyethanol and counted in dioxane-based scintillant.

Adenylate cyclase was assayed in the synaptosomal membranes by incubating for 20 min at 37° 0.3 mg of SPM protein in medium containing, to a total volume of 1 ml, 50 mM Tris–HCl, pH 7.5, 0.5 mM ATP, 5 mM $MgSO_4$, 1 mM EDTA, 5 mM theophylline, 5.6 mM phosphoenolpyruvate (PEP) and 2.3 units of pyruvate kinase. The incubation reaction was stopped by boiling the mixtures for 3 min and the amount of cyclic AMP produced was assayed thereafter in aliquots of supernatants from the incubation mixtures (15 min, 1,500 *g*) by using a modification of the competitive protein binding technique [7], as described elsewhere [8].

At concentrations of free cholesterol and of cholesterol-accepting sites on the membrane of the same order of magnitude, the incorporation of cholesterol in SPM followed second order kinetics, as deduced from equations fitting the experimental data. The number of cholesterol-accepting sites on the synaptosomal membrane was estimated to be 128 ± 20 nmoles per mg of protein. This value was assessed from estimates of the indigenous cholesterol (C) [9] and phospholipid (P) [10] levels in SPM, which gave the figures 552 ± 40 nmoles/mg prot. and 690 ± 60 nmoles/mg prot., respectively (C/P = 0.82) and assuming that the maximum allowable amount of cholesterol that can be incorporated in the membrane satisfies the condition C/P = 1 [11]. Under the conditions of incubation used and due to the limitations inherent in the insolubility of cholesterol in aqueous media and in the prolonged time of incubation, saturation of the incorporation could not be achieved. In fact, only a small percentage (<5%) of the limited number of cholesterol-accepting sites, that defines the condition C/P = 1, participated in the incorporation.

The capacity of such cholesterol-treated membranes to take up 5-HT onto the putative 5-HT receptors was examined within the range of neurotransmitter concentrations from 5 to 1,000 nM. Estimates of uptake of 5-HT in non-treated membranes (Fig. 1) under these conditions (linear uptake range) reflect a high degree of true receptor binding. This is suggested also by the observation (Fig. 1) that 5,6-DHT, a well known potent 5-HT antagonist [12–15], at a concentration of 10 μM reduced 5-HT uptake by SPM significantly. In membranes pre-treated with cholesterol the uptake of 5-HT was reduced by nearly 40 per cent (Fig. 2), compared to non-treated membranes. These data suggest that external cholesterol, entering the structure of the synaptosomal membrane, has, to a certain extent, an effect on membrane serotonergic receptor activity. This suggestion is supported by evidence (Fig. 3) that the activity of

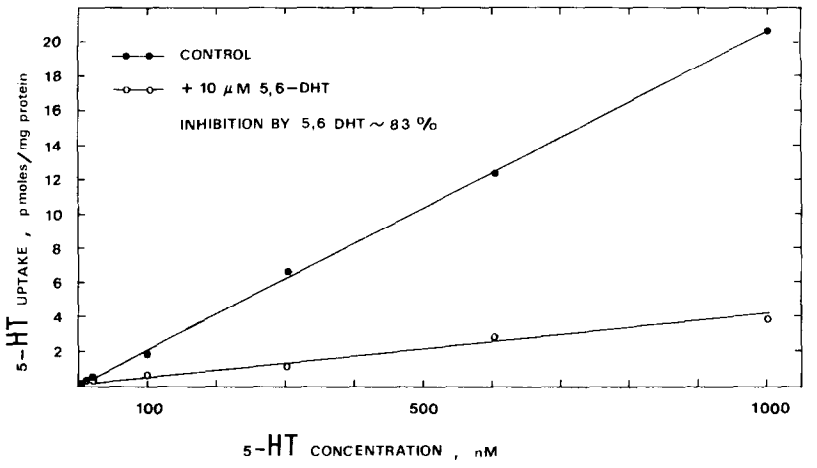

Fig. 1. Uptake of [^{3}H]-5-HT by untreated synaptosomal membranes from dog brain. The drawn curves represent a typical experiment which has been repeated three times and are linear regressions through points which are mean values of duplicate estimates ($r^2 > 0.98$). Background levels at each of the experimental points, corresponding to zero-time incubation, were calculated from a "blank" regression line and were subtracted accordingly. Intercepts calculated from the individual lines have also been subtracted.

SPM-associated adenylate cyclase, a member of the enzymatic complex involved in the physiological propagation of neurotransmitter message in the nervous synapse [16, 17], is also reduced considerably in membranes pre-treated with cholesterol. In this figure, the sensitivity of the synaptosomal membrane, as reflected in the activity of adenylate cyclase, to a minor loading with cholesterol, beyond the indigenous membrane cholesterol level, is evident. This result is in accordance with previously reported information that cholesterol from cholesterol-containing liposomes inhibits the activity of adenylate cyclase in cultured mammalian fibroblasts [18]. It has been postulated that cholesterol may have a role in regulating adenylate cyclase activity [19, 20]. Preliminary results (not shown in this communication) indicate that cholesterol also affects dopaminergic and noradrenergic activities in the isolated synaptosomal membranes from dog brain.

The known asymmetry of structural lipid framework in most biological membranes [21, 22] dictates the thermodynamics of membrane geometry changes and temperature dependence in the steroid–membrane interactions [23]. This may provide, conceivably through an asymmetric distribution of lateral pressure increases, the physical–chemical basis for the observed high degree of sensitivity of the tested membranes to cholesterol entering the lipid bilayer phase. An explanation of the accentuation of these effects,

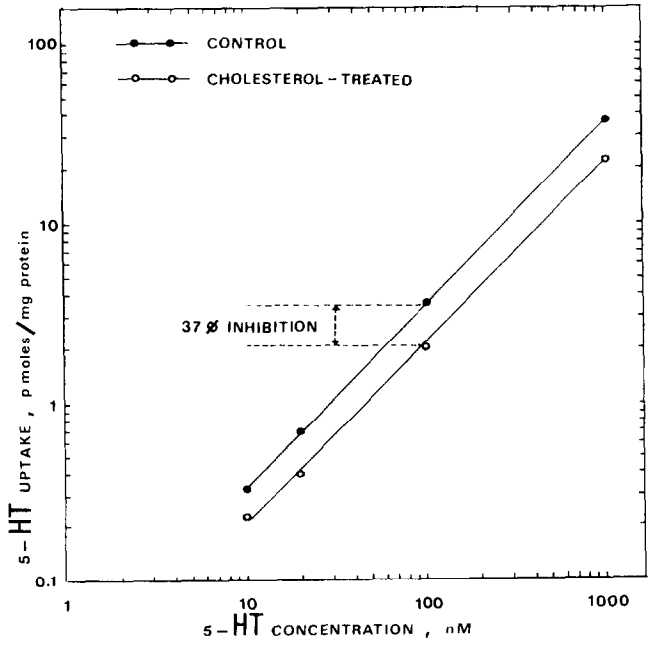

Fig. 2. Uptake of [^{3}H]-5-HT by cholesterol-treated synaptosomal membranes from dog brain. Points in the drawn curves are mean values of duplicate determinations from a typical experiment which has been repeated three times. In all experiments the inhibition of 5-HT uptake by cholesterol was over 35% of the control uptake.

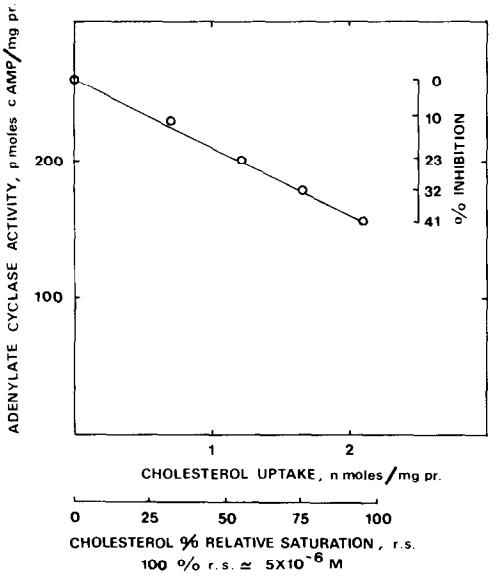

Fig. 3. The activity of SPM-associated adenylate cyclase as a function of cholesterol uptake by the membrane. Membranes were pre-incubated with varying concentrations below saturation of aqueous, buffered cholesterol solutions, as described in the text. In parallel experiments the use of [^{14}C]cholesterol allowed to estimate cholesterol uptake by SPM under similar conditions. The individual variations in estimates of cAMP levels between duplicates in the same experiment were within ± 5% from the mean values. In this figure, average estimates of cAMP from three separate experiments have been related to mean values of estimates of incorporated labeled cholesterol, obtained from two separate experiments each in duplicate determinations.

as required for a high degree of membrane sensitivity to cholesterol, may be based on the recent evidence from this laboratory that there may exist an allosteric cooperativity in cholesterol incorporation in SPM [24]. This is, however, not clearly established at present (imperfect Hill plots of binding) due to the limitations mentioned (inaccessibility of the saturation levels) in this type of experimentation. Nevertheless, the high sensitivity observed *in vitro* of the synaptosomal membrane to cholesterol, as expressed in neurotransmitter activity changes reported in this communication, merits close consideration. It may be a causative factor *in vivo* for deviations from normal neuronal function, which are associated with senescence. Work is now in progress in this laboratory in the direction of investigating effects of cholesterol on the activity of cyclic AMP-dependent membrane-associated protein kinase.

In summary, minor amounts of exogenous cholesterol, incorporated in the structure of the synaptosomal plasma membrane of dog, reduced appreciably the uptake of 5-HT by membrane-associated 5-HT acceptors. The data on the effect of the antagonist 5,6-DHT suggest that in the range of used neurotransmitter concentrations cholesterol alters, to a certain extent, true 5-HT receptor activity. As neurotransmitter receptors are functionally connected with SPM-associated adenylate cyclase, the observed changes induced by cholesterol on the activity of this enzyme also suggest that exogenous cholesterol may affect, more generally, the mechanism associated with neurotransmitter message propagation in the neuronal synaptic junction.

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