Biochimica et Biophysica Acta, 307 (1973) 467–470

BBA 76347

TETRODOTOXIN–CHOLESTEROL INTERACTIONS AT THE AIR–WATER INTERFACE

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(Received December 29th, 1972)

SUMMARY

A claimed, specific interaction of tetrodotoxin with cholesterol monolayers could not be repeated. An observed expansion of cholesterol monolayers on a subphase of the toxin in citrate and phosphate buffer was shown to be time dependent and probably due to a surfactant impurity of the phosphate salts.

INTRODUCTION

Earlier work by Villegas *et al.*[1] suggested that because tetrodotoxin interacted specifically with monolayers of cholesterol, cholesterol might be an integral component of the sodium channel of nerve cells. In the light of Hille's[2] recent data and reasoning, any such claim required substantiation. Although no difficulty was encountered in confirming an expansion of a cholesterol monolayer on a substrate containing tetrodotoxin, we were also able to demonstrate an equal effect on a simple, tetrodotoxin-free, phosphate salt solution.

EXPERIMENTAL

All water used was tri-distilled, the final distillation being in the presence of alkaline $KMnO_4$. Cholesterol (Δ^5-cholesten-3-ol) was obtained from Sigma Chemicals as the >99% pure standard for chromatography and was dissolved in alumina-purified 60–80 petroleum ether. All other chemicals were Analytical Reagent grade. The tetrodotoxin was obtained from Sankyo Co. Ltd. Tokyo, Japan as a lyophilized powder in citrate buffer.

A Teflon trough of 100 ml capacity, 20 cm × 6 cm and 0.8 cm deep had a sliding Teflon piston/barrier to facilitate changes in surface area. The barrier was connected directly to the X-axis of a Bryans 21001 X–Y potentiometric recorder which was itself activated by a reversible, voltage ramp generator. A platinised platinum dipping wire, diameter 0.31 mm, suspended from a Mark I micro-Force Balance (C.I. Electronics Ltd) with direct reading output was used to Monitor changes in surface pressure. The changes were recorded on the Y-axis of the recorder and surface pressure–area curves were thus automatically obtained. A micro switch was positioned so that the ramp generator, activating the X-axis, would reverse at a predetermined surface

pressure, to return at the same rate as in the forward direction. Micro switches were also positioned at the limit of the barrier travel so that the dipping wire would not be damaged by overrunning of the piston/barrier. Two types of piston/barrier were used; the first was flat and gave a compression ratio of 6.3:1. The second had a recess of 1.5 cm × 1 cm cut into the forward face, which enabled a greater compression ratio to be obtained, namely 10.7:1. The latter was only used when indicated in the text.

Homogeneity of the subphase after an addition of reagent was ensured by a rotating, glass-sheathed magnetic stirrer which travelled back and forth along the length of the trough. The whole apparatus was situated in a cupboard to eliminate draughts and dust particles. An Agla micrometer syringe was used to apply 10 μl of approximately 4 mM cholesterol in 60–80 petroleum ether to the air–water interface. Subphases were chosen to cover a range of concentrations of phosphate buffer (pH 7.0) in 0.15 M NaCl and blanks were also run on pure water. Tetrodotoxin citrate or the sodium citrate equivalent (100 μg/ml) was added and stirred into the subphase in 100-μl quantities using a 500-μl Hamilton syringe after the monolayer had been applied and while the monolayer was still fully extended. Monolayers were only compressed when a reading was required and after the subphase had been stirred. The biological activity of tetrodotoxin was measured according to the method of Evans[3] and was always found to be fully active. The area per molecule A_0, is taken as the area that would be occupied by one molecule at zero pressure, if the linear region of the π–A curve, when the monolayer is highly incompressible, is extrapolated to zero pressure[4]. Our sample of cholesterol consistently gave values of 39.7 Å^2 on water at 22 °C.

RESULTS AND DISCUSSION

Fig. 1 shows that both tetrodotoxin-containing citrate and a tetrodotoxin-free sodium citrate equivalent have an expanding effect on a cholesterol monolayer if a subphase of 0.15 M NaCl and 10 mM sodium phosphate buffer, pH 7.4, is used. In this experiment successive 100-μl aliquots of the solutions (100 μg/ml) were added through the monolayer into the subphase which was then stirred for a few minutes before a reading was taken. The temperature was approximately 22 °C and the error bars marked, are those inherent in reading the results from the original plots. It would appear that the sodium equivalent solution of tetrodotoxin is capable of producing

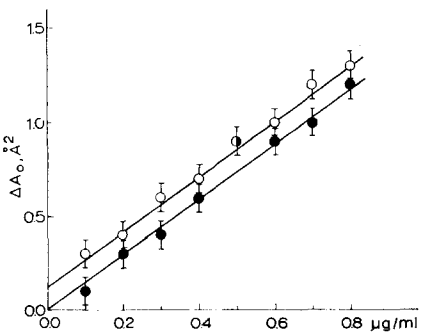

Fig. 1. Comparison of apparent change in A_0 of cholesterol monolayers as a function of subphase concentrations of tetrodotoxin (○) and tetrodotoxin equivalent (●), using successive 100-μl additions.

almost the same effect as that containing tetrodotoxin. Several experiments stemmed from this result, the first of which was to study an ageing effect of cholesterol monolayers on a tetrodotoxin-free, sodium citrate equivalent over a period of 2 h. The results are shown in Fig. 2 where it can be seen that there is a time-dependent increase in the area/molecule of cholesterol which could have accounted for the displacement

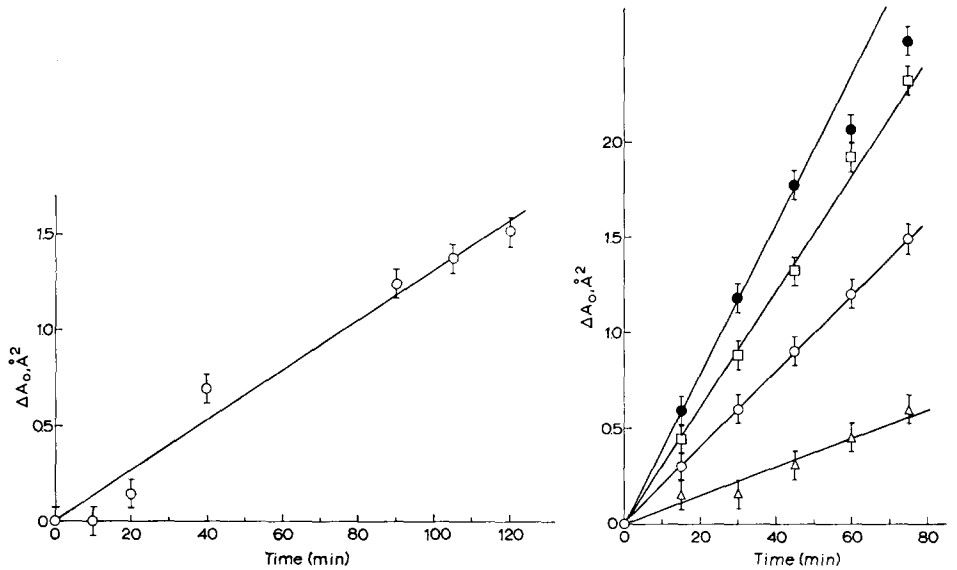

Fig. 2. Time-dependent apparent change in A_0 of cholesterol monolayers spread on tetrodotoxin equivalent subphases.

Fig. 3. The effect of different subphases, pure water (△) 25 mM (○), 50 mM (□), and 75 mM (●) phosphate buffer, pH 7.0, on the apparent expansion of cholesterol monolayers.

of the tetrodotoxin-free curve depicted in Fig. 1. In a second experiment cholesterol monolayers were spread on differing concentrations of phosphate buffer only, all at pH 7.0 and the effect of ageing on the monolayer noted. The results may be seen in Fig. 3 and lead to the conclusion that phosphate alone is the principle cause of the apparent increase in the area per cholesterol molecule. Whether this reaction is one catalysed by phosphate, a binding of phosphate to cholesterol or merely some impurity in the phosphate salt, is not known. In the belief that the phenomenon was a time-dependent accumulation of an air–water surface-active material, a third set of experiments was carried out as follows: The simple, straight piston/barrier was substituted by the one having the small rectangular recess cut in the middle of the limiting edge. Into this recess the dipping wire could be located centrally whilst the leading edge closed with the end of the trough thereby concentrating any surfactant molecules by approximately 10-fold.

When 75 mM phosphate only was present as a subphase and the surface area of the accumulated surfactant measured, condensed material occupying at least 4.5 cm^2 was invariably present after some 75 min. This value is to be compared with an apparent gain of 5.0 cm^2 in total surface area with cholesterol on a similar subphase, for a similar time. Indeed, a time curve for the area accumulation of the unknown

surfactant and the area increase for a cholesterol monolayer is rather similar. See Fig. 4.

A recent paper by Kamel *et al.*[5] also gives results which question the validity of Villegas' work. They note that cholesterol is oxidized at an air–liquid interface when 65 mM phosphate buffer, pH 7.0, is used. An expansion of approximately 12 Å^2 per molecule was observed after 60 min due to the formation of a mixture of the epimeric

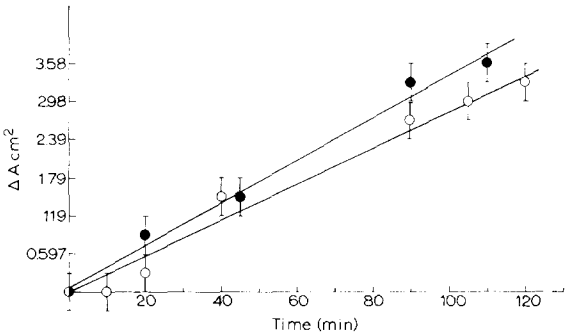

Fig. 4. Comparison of increase of total monolayer area ΔA with (○) and without (●) cholesterol monolayers on 75 mM phosphate buffer, pH 7.0, as a function of time.

7-hydroxy cholesterols, 7-keto cholesterol and other products. Although we never recorded such a degree of expansion per molecule of cholesterol, there are two interesting points to note. Firstly, when any of the oxidation products were left on 65 mM phosphate buffer, pH 7.0, for 60 min a further expansion of about 2 Å per molecule was observed. As this gain was not explained by oxidation, might it not be an impurity leaching out from the phosphate buffer in the manner described above? Secondly, when the oxidized cholesterol monolayers were analysed by thin-layer chromatography, a large spot of unidentified material was always present.

Although the presented evidence is only circumstantial, the important point is that both Kamel *et al.*[5] and ourselves find a time-dependent increase in the area per molecule of cholesterol, when spread as a monolayer on a phosphate buffer. This expansion is of the same order of magnitude as that claimed to be caused specifically by tetrodotoxin.

We have thus been unable to show any significant interaction between tetrodotoxin and cholesterol (Fig. 1), and are therefore unable to explain the results of Villegas *et al.*[1] with our measurement. Indeed, their reported concentration dependence of the expansion of the monolayer is in contradiction to our results. It is possible, but we consider unlikely, that the method chosen by the present authors together with the unknown contaminant, masked the reported interaction.

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