Biochimica et Biophysica Acta, 291 (1973) 61–70

BBA 76086

FILIPIN AS A FLUORESCENT PROBE FOR THE LOCATION OF CHOLESTEROL IN THE MEMBRANES OF FRAGMENTED SARCOPLASMIC RETICULUM

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(Received May 3rd, 1972)
(Revised manuscript received July 31st, 1972)

SUMMARY

A polyene antibiotic, filipin, when excited at 360 nm in solvents of low polarity, exhibits a strong fluorescence with a maximum at 480 nm. Mixed lecithin–cholesterol micelles, but not aqueous dispersions of cholesterol, also cause an increase of filipin fluorescence.

Filipin binds to the vesicles of fragmented sarcoplasmic reticulum. The binding is considerably decreased after removal of cholesterol and abolished after lipid depletion. A restoration of filipin binding is achieved in the former case by rebinding of cholesterol, in the latter case only by the cholesterol–phospholipid micelles. Cholesterol additionally bound to intact vesicles does not cause an increase of binding of filipin. An enhancement of filipin fluorescence intensity is observed on its binding to the fragmented sarcoplasmic reticulum, indicating binding to regions of low polarity.

Fluorescence with a maximum at about 340 nm, exhibited by sarcoplasmic reticulum vesicles by excitation at 280 nm, decreases in the presence of filipin with a concomitant appearence of fluorescence with a maximum at 480 nm. These results indicate an energy transfer from tryptophan residues to filipin.

INTRODUCTION

Our recent studies on the role of cholesterol in the specific function of fragmented sarcoplasmic reticulum, *i.e.* in the ATP-dependent accumulation of Ca^{2+}, were mainly devoted to the use of solvent systems enabling a selective removal of this lipid[1]. Previous studies by van Deenen and collaborators[2–5] and other research workers[6,7], performed on lipid mono- and bilayers, liposomes and erythrocyte membranes, showed that filipin, a neutral polyene antibiotic[8], interacts with cholesterol. In this work we used filipin to study the possible effect of its interaction with cholesterol present in the fragmented sarcoplasmic reticulum on the specific properties of these biomembranes. The presence of a conjugated double bond system in filipin implied that this compound should exhibit a strong fluorescence in apolar media[9], as

also suggested by Wallach *et al.*[10]. Since this was found indeed to be the case, we used this antibiotic as a fluorescence probe for investigations of some features of the binding of cholesterol to the membranes of fragmented sarcoplasmic reticulum. In one part of the study the fluorescence spectra were examined during excitation in the regions of absorbance peaks of filipin in order to obtain information on the hydrophobicity of the environment of bound cholesterol interacting with filipin. Since the absorbance peaks of filipin overlap the region of fluorescence emission of protein chromophores, the transfer of excitation energy[10,11] from membrane protein to filipin bound to cholesterol was also studied.

A preliminary report of this work was presented at the 7th FEBS Meeting in Varna, 1971 (ref. 12).

MATERIAL AND METHODS

Preparation of fragmented sarcoplasmic reticulum

Sarcoplasmic reticulum vesicles were prepared from rabbit leg and back skeletal muscle and purified on a continuous sucrose density gradient[1]. ATPase activity and Ca^{2+} uptake were measured as described previously[1]. Vesicles devoid of cholesterol were prepared by extraction of lyophilized vesicles with dry ethyl ether[1]. Lipid-depleted vesicles were obtained by the procedure of Fleischer and Fleischer[13] with 90 % acetone.

Preparation of lipid dispersions

From the solutions of lecithin, cholesterol, or both lipids in chloroform, the solvent was evaporated under N_2 and the lipid dispersions were prepared by a 2-min sonication with cooling in 0.1 M KCl and 20 mM histidine (pH 7.2), with the use of a sonifier (MSE Ltd, London). Control experiments with the use of ^{14}C-labelled cholesterol revealed that the cholesterol dispersions obtained in this way were homogeneous, even in the absence of lecithin, since after centrifugation for 1 h at 105000 $\times$ *g* no concentration gradient was formed.

Protein concentration was determined by the biuret method[14] with bovine plasma albumin as a standard. Fluorescence spectra were recorded on an Aminco–Bowman spectrophotofluorimeter equipped with a high pressure Xe–Hg lamp and an RCA 1P 28 photomultiplier. Measurements were carried out at 20 °C. All spectra were uncorrected.

Chemical determinations

Egg lecithin was isolated according to the method of Pangborn[15]. Its purity was routinely examined by chromatography on silica gel plates with chloroform–methanol–water (65:35:4, v/v/v). Filipin (U-5956 (crystalline complex), ref. No. 8393-DEG-11-8, potency 86% pure) was a generous gift of Dr G. B. Whitfield, The Upjohn Company, Kalamazoo, Mich., U.S.A. It was protected against exposure to light and stored at −10 °C under N_2. Stock solutions of filipin in dimethylformamide were prepared before each experiment. Final concentration of dimethylformamide in the reaction mixture never exceeded 5 %, unless otherwise stated. ATP and cholesterol were purchased from E. Merck AG-Darmstadt, Germany. All organic solvents were distilled before use.

RESULTS

Fig. 1 presents the excitation spectrum of filipin which shows two major maxima at about 357 and 338 nm; when excited at one of these wavelengths filipin exhibited a fluorescence emission with a maximum at about 480 nm. Fluorescence intensity was low in water and greatly increased in solutions of low polarity (Fig. 2).

The addition of cholesterol dispersions to filipin in water did not increase fluorescence intensity (Fig. 3). Similarly, phospholipid micelles were without effect. On the other hand, mixed phospholipid–cholesterol micelles caused a strong enhancement of filipin fluorescence intensity. These results suggested that only in the case of cholesterol present in phospholipid micelles was there a sufficiently hydrophobic environment favouring an increase of fluorescence of filipin; the results also indicated that filipin may be useful as a fluorescence probe for studying several binding phenomena of cholesterol.

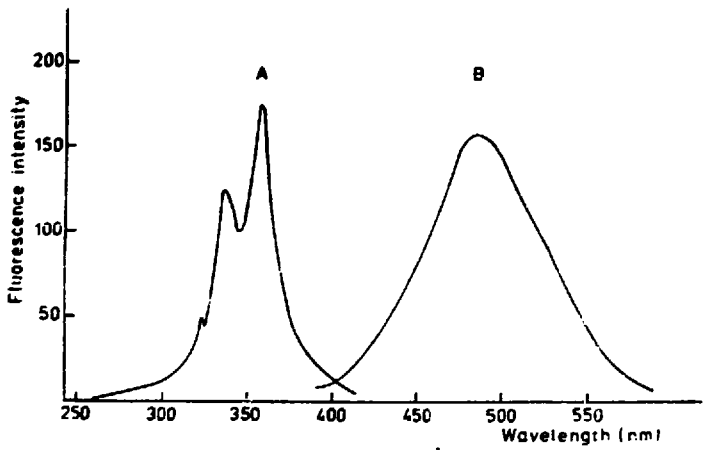

Fig. 1. Excitation (A) and emission (B) spectrum of filipin in dimethylformamide. Emission at 480 nm, excitation at 360 nm.

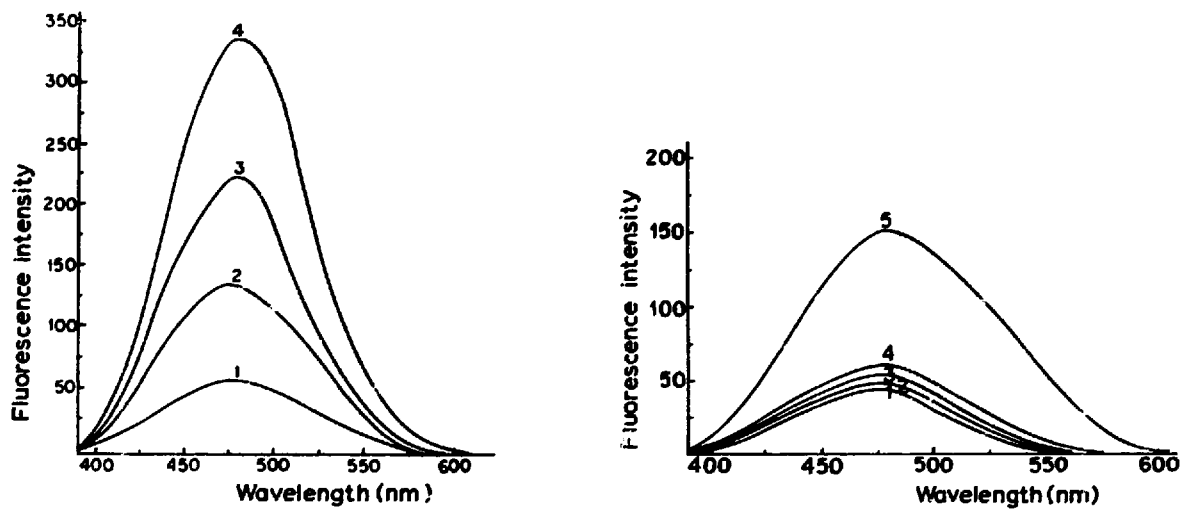

Fig. 2. Emission spectrum of filipin in solvents of different polarity. 0.1 mM filipin in (1) water; (2) ethanol; (3) dimethylformamide; (4) dioxane. Excitation wavelength 360 nm.

Fig. 3. Fluorescence intensity of filipin in the presence of lipid dispersions. All samples contained 0.1 mM filipin. (1) Control, without lipids. Additions: (2) 0.12 mM cholesterol; (3) 1.2 mM cholesterol; (4) micelles of 0.12 mM lecithin; (5) mixed micelles of 0.12 mM lecithin and 0.12 mM cholesterol. All lipids were ultrasonically dispersed in water prior to measurement. Excitation wavelength 360 nm.

When filipin was added to the suspension of sarcoplasmic reticulum vesicles, a considerable enhancement of its fluorescence intensity was observed (Fig. 4), indicating binding of filipin to the regions of low polarity. The relative value of fluorescence intensity depended on the ratio of filipin to the sarcoplasmic reticulum vesicles. When increasing amounts of filipin were added to the same amount of vesicles, the increment of fluorescence showed a biphasic character (Fig. 5). Filipin added in amounts of up to 0.2 μmole per mg vesicular protein caused a much higher

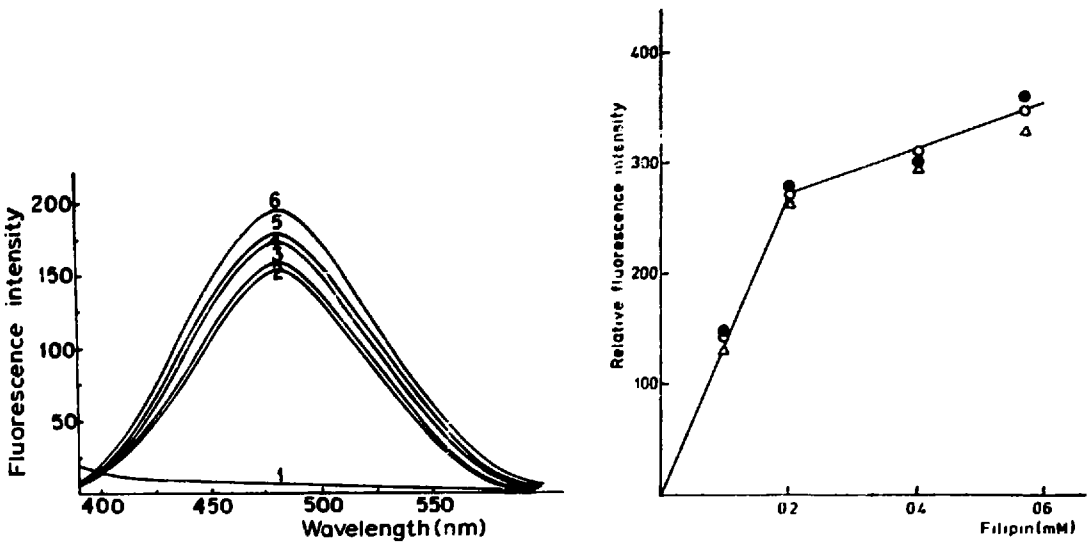

Fig. 4. Fluorescence intensity of filipin in the presence of sarcoplasmic reticulum vesicles. All samples contained vesicles (1 mg protein/ml) in 20 mM histidine (pH 7.2) and 0.1 M KCl. (1) Control, without filipin; (2–6) 0.1 mM filipin and, in addition: (3) 0.12 mM cholesterol; (4) 1.2 mM cholesterol; (5) 0.12 mM lecithin; (6) mixed phospholipid–cholesterol micelles. In the latter case, samples containing vesicles (1 mg protein/ml) and micelles of 0.12 mM lecithin and 0.12 mM cholesterol were incubated at 20 °C for 30 min and then centrifuged at 105000 × g for 30 min. Pellets after resuspension to the concentration of 1 mg protein/ml were used for measurement.

Fig. 5. Relationship between concentration of filipin and its fluorescence in the presence of vesicles. All samples contained vesicles (1 mg protein/ml) and filipin as indicated on the abscissa. On the ordinate the increment of fluorescence intensity of filipin in the presence of vesicles over that obtained in water is presented. Different symbols denote different experiments.

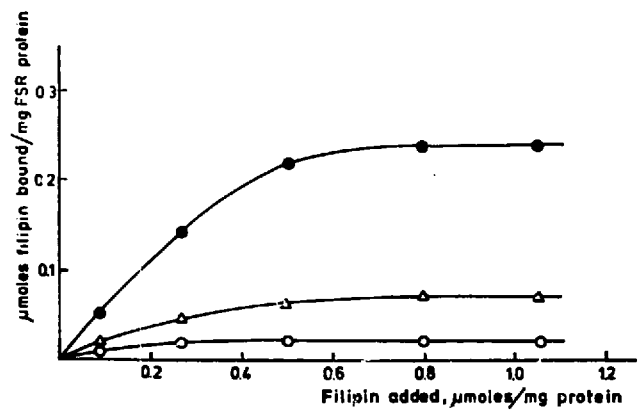

Fig. 6. Binding of filipin to sarcoplasmic reticulum vesicles. Samples containing vesicles (1 mg protein/ml) in 20 mM histidine (pH 7.2), 0.1 M KCl and filipin in concentrations indicated on the abscissa were incubated at 20 °C for 30 min and then centrifuged at 105000 × g for 30 min. Filipin bound to vesicles was calculated from the amount of filipin remaining free, determined in the supernatant with the use of a molar extinction coefficient given by ref. 8. ●, intact vesicles; △, vesicles devoid of cholesterol; ○, lipid-depleted vesicles. For details see Methods. FSR, fragmented sarcoplasmic reticulum.

enhancement of fluorescence than higher concentrations of this antibiotic, which produced only a very small further increase of fluorescence. For calculation of the relationship between the enhancement of fluorescence by filipin and the amount of cholesterol present in sarcoplasmic reticulum membranes, the binding of this antibiotic to the vesicles was determined. Vesicles after incubation with various amounts of filipin were centrifuged and filipin remaining free in the supernatant was determined. Fig. 6 shows that intact vesicles bind up to about 0.25 μmole filipin per mg protein, an amount about twice as high, on a molar basis, as the amount of cholesterol present in the vesicles (about 0.12 μmole per mg protein[1]). When 0.2 μmole of filipin was added per mg vesicular protein, *i.e.* at the break-point of the curve in Fig. 5, about half the filipin was bound to the vesicles (Fig. 6). If the first part of the curve reflects binding of filipin to cholesterol, the obtained data suggest that filipin interacts with that lipid in a molar ratio. Consequently, the amount of filipin added (0.2 μmole) should be sufficient to saturate all cholesterol. Filipin added in excess of this amount causes only a slight additional enhancement of fluorescence (see Fig. 5), which may indicate interaction with phospholipids. This view is supported by the observations on the effect of filipin on specific properties of sarcoplasmic reticulum fragments, *i.e.* Ca^{2+} uptake and Ca^{2+}-dependent ATPase activity (Fig. 7). In accordance with previous observations[16,17], lower concentrations of filipin have no effect on the Ca^{2+}-storage ability of vesicles; under these conditions filipin interacts with cholesterol. As shown recently by us[1], this lipid is not directly connected with the specific properties of sarcoplasmic reticulum. On the other hand, concentrations of filipin higher than 0.2 μmole/mg protein inhibit both Ca^{2+} uptake and ATPase activity. This can be explained by the interaction of filipin with phospholipids. As already well established, phospholipids are essential for the activity of fragmented sarcoplasmic reticulum[18].

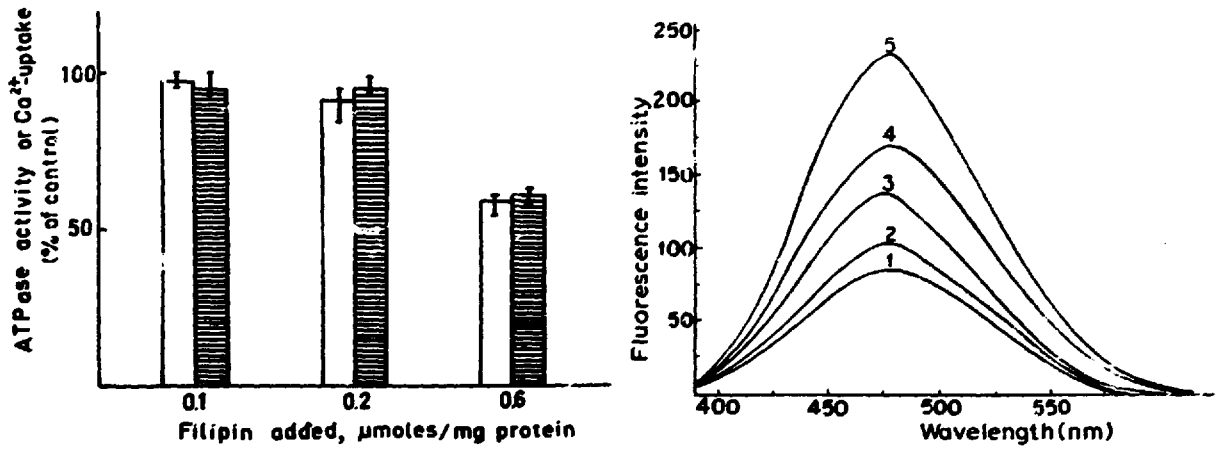

Fig. 7. Effect of filipin on the ATPase activity and Ca^{2+} uptake of vesicles. Samples of sarcoplasmic reticulum vesicles and filipin in concentrations as indicated were incubated at 20 °C for 30 min and centrifuged at 105000 × *g* for 30 min. Pellets were resuspended and ATPase activity (empty bars) and Ca^{2+} uptake (dashed bars) were measured in a medium containing 0.1 M KCl, 20 mM histidine (pH 7.2), 2.5 mM potassium oxalate, 2.5 mM $MgCl_2$, 2.5 mM ATP, 0.1 mM $^{45}CaCl_2$, as previously described[1]. In the figure the results of three experiments carried out on different vesicles preparations are presented. The values are depicted as the mean ± the range.

Fig. 8. Effect of lipids on fluorescence intensity of filipin in the presence of vesicles devoid of cholesterol. All samples contained vesicles devoid of cholesterol (1 mg protein/ml), 0.1 mM filipin and, in addition: (1) none; (2) 0.12 mM lecithin; (3) 0.12 mM cholesterol; (5) 1.2 mM cholesterol. For sample (4) the vesicles contained, in addition, bound phospholipid–cholesterol micelles obtained by ultracentrifugation of vesicles with micelles as described in legend to Fig. 4.

Our recent studies[1] showed that cholesterol could be bound to the intact sarcoplasmic reticulum vesicles in amounts of up to at least 1 mg per mg of vesicular protein. One can see in Fig. 4 that cholesterol additionally bound to vesicles caused only a very slight increase of fluorescence intensity. Control experiments showed that this extra bound cholesterol did not increase considerably the binding of filipin to the vesicles (Table I). Similarly, only a small increase of filipin fluorescence intensity was observed when phospholipid micelles were added to the sarcotubular vesicles. Somewhat higher enhancement of fluorescence was found in the case of mixed lecithin–cholesterol micelles (Fig. 4). In these experiments, the unbound lipids were removed by centrifugation so that only the effect of those cholesterol–lecithin micelles which were bound to the vesicles was examined.

After removal of cholesterol by treatment of lyophilized vesicles with dry ethyl ether[1], the binding of filipin was considerably reduced (Fig. 6) and fluorescence intensity was very low (Fig. 8). Under these conditions filipin was most probably bound to phospholipids. Cholesterol added back to the vesicles caused an enhance-

TABLE I

EFFECT OF ADDED CHOLESTEROL ON THE BINDING OF FILIPIN TO SARCOPLASMIC RETICULUM VESICLES

Samples containing vesicles (1 mg protein/ml) and ultrasonically dispersed cholesterol in concentrations as indicated were incubated at 20 °C for 30 min and then centrifuged at 105000 × *g* for 30 min. Pellets were suspended to the concentration of 1 mg protein/ml, incubated with filipin and again centrifuged in a Spinco ultracentrifuge. Other experimental conditions as described in legend to Fig. 6.

Filipin added (*mM*)	*Filipin bound* (*mM*)		
Cholesterol added (*mM*):	*None*	*0.1*	*1.0*
0.09	0.05	0.06	0.07
0.18	0.08	0.10	0.12

TABLE II

EFFECT OF CHOLESTEROL AND LECITHIN ON THE BINDING OF FILIPIN TO LIPID-DEPLETED VESICLES

Binding of cholesterol and lecithin to vesicles lipid-depleted according to Fleischer[13] and binding of filipin was determined as described in Table I.

Vesicles	*Addition* (*μmoles per mg protein*)		*Filipin* (*μmoles per mg vesicular protein*)	
	Cholesterol	*Lecithin*	*Added*	*Bound*
Intact	0	0	0.20	0.09
	0	0	0.50	0.23
Lipid-depleted	0	0	0.20	0.01
	0.12	0	0.20	0.02
	1.20	0	0.20	0.03
	0.12	0.12	0.20	0.07
	0	0	0.50	0.01
	0.12	0	0.50	0.03
	1.20	0	0.50	0.03
	0.12	0.12	0.50	0.20

ment of filipin fluorescence intensity, although somewhat higher concentrations of this lipid were needed to achieve the level of fluorescence observed with intact vesicles. A slightly higher fluorescence intensity was observed with mixed phospholipid–cholesterol micelles bound to the membranes devoid of cholesterol (Fig. 8).

After removal of lipids from the vesicles with 90% acetone[13], only minute amounts of filipin were bound (Fig. 6 and Table II). The rebound cholesterol did not restore binding nor, consequently, the filipin fluorescence, unless it was added in the form of micelles with phospholipids (Table II and Fig. 9). In the latter case, similar to the experiments discussed above, unbound micelles were removed by centrifugation.

The lack of absorbance of filipin in the regions of 280 nm and the peak of absorbance at 340 nm, *i.e.* in the region of emission of tryptophan fluorescence, suggested the possibility of energy transfer from membrane protein chromophores to filipin, provided its binding sites, *i.e.* cholesterol molecules, were located close enough to the tryptophan residues. Sarcoplasmic reticulum vesicles, when excited at 280 nm in the absence of filipin, exhibited fluorescence with a maximum at 340 nm characteristic for tryptophan in hydrophobic environment (Fig. 10), (see also Vanderkooi and Martonosi[19]). Addition of increasing concentrations of filipin caused a progressive decrease of fluorescence at 340 nm, with the appearance of fluorescence emission with a maximum at 480 nm typical for filipin. No fluorescence at 480 nm was observed in the solutions containing tryptophan, lecithin–cholesterol micelles and filipin after excitation at 280 nm.

Lipid-depleted vesicles, when excited at 280 nm, show somewhat lower fluorescence at 340 nm than untreated ones. Although the rebound cholesterol–lecithin micelles, when excited at 360 nm (see Fig. 6), enhanced the filipin fluorescence, they did not cause any fluorescence emission at 480 nm by excitation at 280 nm, indicating a lack of energy transfer from tryptophan to filipin in this case.

Electron micrographs of negatively stained preparations of fragmented sarcoplasmic reticulum, treated with filipin in concentrations higher than 0.3 mM, indicated

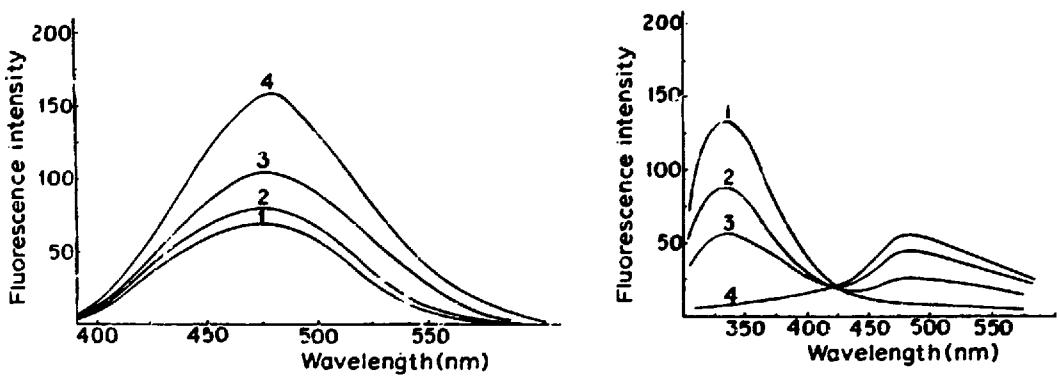

Fig. 9. Effect of lipids on fluorescence intensity of filipin in the presence of lipid-depleted vesicles. All samples contained lipid-depleted vesicles (1 mg protein/ml), 0.1 mM filipin and in addition: (1) none; (2) 1.2 mM cholesterol; (3) 0.12 mM lecithin. For sample (4) the vesicles contained, in addition, bound phospholipid–cholesterol micelles (for details of the procedure see legend to Fig. 4).

Fig. 10. Emission spectrum of membranes of sarcoplasmic reticulum vesicles. All samples contained vesicles (1 mg protein/ml). (1) Vesicles alone. (2–4) Filipin added: (2) 0.1 mM; (3) 0.2 mM; and (4) 0.4 mM. Excitation wavelength 280 nm.

the appearance of modified vesicles. Preliminary observations seem to indicate that the first step is the fragmentation of vesicles into small particles with a diameter of about 400 Å, followed by their aggregation into a very specific regular pattern (Fig. 11).

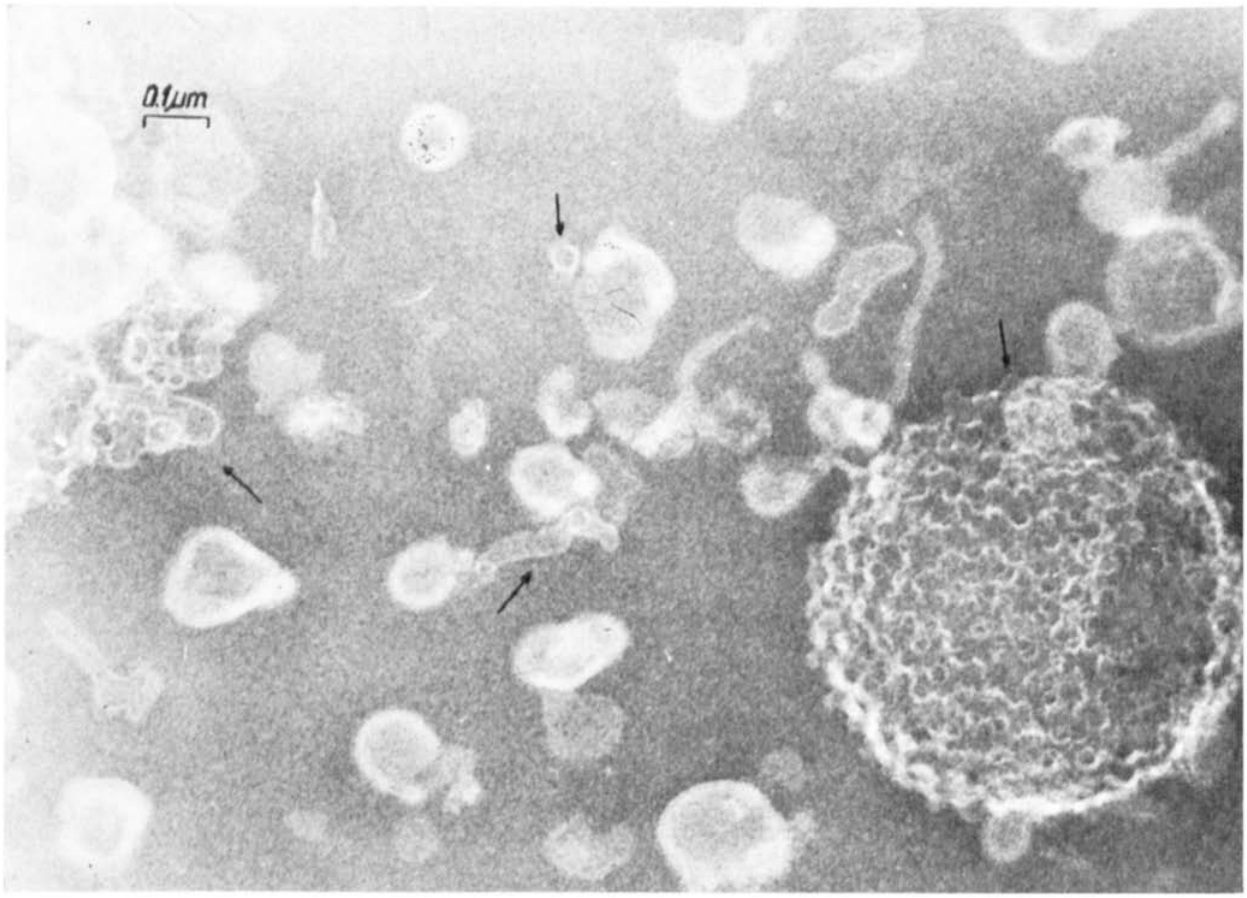

Fig. 11. Electron micrograph of the vesicles of sarcoplasmic reticulum treated with 0.5 mM filipin and negatively stained with 2% phosphomolybdate as previously described[1]. Modified vesicles indicated by arrows. Magnification: 62000 ×.

DISCUSSION

The results of this work, performed on vesicles of fragmented sarcoplasmic reticulum, confirm previous observations[2–7] indicating that the essential site of filipin binding in biomembranes is cholesterol, but that, at higher concentrations of filipin, binding to phospholipids[4–7] also takes place. The effect of filipin on the properties of fragmented sarcoplasmic reticulum, depending on the amount used and, consequently, on the kind of interacting lipid, is in agreement with that view. As in the treatment with various other agents, interaction with cholesterol does not affect the specific properties of fragmented sarcoplasmic reticulum, but interaction with phospholipids abolishes them.

The present results also show that filipin does not interact with cholesterol if that lipid is bound to the vesicles in the form of sonicated dispersions. One may argue that, in the case of lipid-depleted vesicles, the attachment and/or localization of rebound cholesterol is different to that in intact vesicles, probably due to denaturation of proteins by lipid depletion. However, dispersed cholesterol additionally bound to

intact vesicles also only slightly enhances the binding of filipin. Hence, one can assume that, in all these cases, the kind of dispersion of cholesterol molecules used does not favour interaction with filipin. Consequently, the lack of enhancement of fluorescence by cholesterol dispersions obtained in the absence of phospholipids (Fig. 3) can be, in fact, due to the lack of interaction with filipin.

On the other hand, cholesterol in the form of micelles with phospholipids interacts with filipin even when bound to lipid-depleted vesicles. Whether the role of phospholipids is only to provide the proper dispersion of cholesterol molecules necessary for interaction with filipin, or whether in the absence of phospholipids filipin does not interact with cholesterol in any case, remains to be elucidated.

The present results also show that filipin in an apolar medium exhibits strong fluorescence and that this property can be utilized to study several aspects of cholesterol binding to various biomembranes. In the model systems cholesterol enhances filipin fluorescence when it is "dissolved" in phospholipids. Those results, together with those of the binding experiments discussed above, indicate that the hydrophobic environment caused by phospholipids is a necessary prerequisite of the binding of filipin to cholesterol. In agreement with the current view on the location of cholesterol in biomembranes[20,21], this requirement is also fulfilled in the case of cholesterol originally present in the membranes of sarcoplasmic reticulum, as indicated by the binding of filipin by the vesicles and the enhancement of its fluorescence.

The other advantage of filipin as a fluorescence probe is its use for studies on the energy transfer from membrane protein chromophores. The results of this work clearly show that in the membranes of sarcoplasmic reticulum an energy transfer from tryptophan to filipin takes place. As pointed out recently by Wallach *et al.*[10], lipophilic fluorescence probes can be very useful for studies of protein–lipid interactions in the biomembranes for the calculation of the proximity of these components. The results of this work show that the quenching of tryptophan fluorescence by filipin is large, hence the donor–acceptor distance should be small, indicating that at least part of the cholesterol molecules are located in close vicinity to the residues of this amino acid. A similar energy transfer from tryptophan to 8-anilino-1-naphthalene sulphonate bound to sarcotubular membranes was recently observed[19].

Work is now being carried out in order to obtain more details of the phenomenon of energy transfer from tryptophan to filipin, and a quantitative evaluation will be published elsewhere.

Contrary to intact vesicles, lipid-depleted ones, even after rebinding of cholesterol–lecithin micelles, do not exhibit an energy transfer from tryptophan to filipin, suggesting, in this case, a different location of rebound lipids, probably due to irreversible conformational changes in the membrane proteins caused by treatment with acetone during lipid depletion. It has already been shown earlier that this procedure irreversibly destroys the specific properties of fragmented sarcoplasmic reticulum[18].

Kinsky *et al.*[3] observed earlier in the electron microscope that filipin causes formation of "pits" in erythrocyte membranes and lecithin–cholesterol dispersions. However, the morphological alterations brought about by this antibiotic in the membranes of fragmented sarcoplasmic reticulum are different. The reason for this is now under investigation.

ACKNOWLEDGEMENTS

The authors are extremely grateful to Prof. D. Shugar of the Department of Biophysics, University of Warsaw, for making available a spectrofluorimeter and to Dr M. Zaborowska for the measurements, as well as to Dr A. Wroniszewska for electron micrographs of filipin-treated vesicles. The skilled technical assistance of Mrs A. Pawlak is acknowledged.

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