Volume 288, number 1,2, 237–240 FEBS 10083 August 1991
 00145793/91/$3.50
ADONIS 001457939100796Y

Characterization of the interaction of doxorubicin with (poly)phosphoinositides in model systems

Evidence for specific interaction with phosphatidylinositol-monophosphate and -diphosphate

Frits A. de Wolf[1,2], Rudy A. Demel[2], Danny Bets[2], Carlos van Kats[2] and Ben de Kruijff[1,2]

[1]*Institute of Molecular Biology and Medical Biotechnology*, and [2]*Centre for Biomembranes and Lipid Enzymology, State University Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands*

Received 18 June 1991; revised version received 26 June 1991

The anticancer drug doxorubicin penetrates into Langmuir monolayers containing phosphoinositides. Upon binding of doxorubicin to phosphoinositide-containing SUV, its fluorescence is self-quenched due to self-association. As compared to other anionic phospholipids, as much as 2- to 3-fold larger effects were obtained with PIP and PIP_2, in mixtures of these lipids with DOPC. Doxorubicin competes efficiently with the non-penetrating antibiotic neomycin for binding to PIP_2. According to its penetration, specific binding of doxorubicin was half-maximal at 5–15 μM. It is likely that also in biological membranes doxorubicin binds specifically to PIP and PIP_2.

Doxorubicin; Phosphoinositide; Anionic phospholipid; Drug–membrane interaction; Membrane penetration; Drug stacking

1. INTRODUCTION

Doxorubicin and related anthracycline antibiotics are potent and widely-applied anticancer agents. They interact with DNA and topoisomerase II [1] as well as with membranes. At least in model membrane systems, anionic phospholipids appear to be an important target [2–6]. Using NMR, fluorescence, X-ray and centrifugation techniques, we have recently carried out a systematic comparison of the interactions between doxorubicin and model membranes consisting of various phospholipids, including phosphatidic acid (PA) and phosphatidylserine [6]. The drug appeared to bind specifically to anionic phospholipids, to penetrate into the membrane and to induce a disordering of the acyl chains. Interactions between anthracyclines and anionic phospholipids involved in signal transduction are highly interesting in view of their possible effects on the regulation of cell proliferation [7]. It is therefore surprising that hardly anything is known about the interactions with (poly)phosphoinositides. We presently characterized the interactions of doxorubicin with model membranes containing phosphatidylinositol (PI), phosphatidylinositol-monophosphate (PIP) or -diphosphate (PIP_2).

Correspondence address: F.A. de Wolf, Institute of Molecular Biology and Medical Biotechnology, State University Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands. Fax: (31) (30) 522478.

Abbreviations: EGTA, di-(aminoethyl)-glycolether-*N,N,N',N'*-tetraacetic acid; PIPES, 1,4-piperazine-diethanesulfonic acid; (DO)PA, (SA)PA, (DO)PC, (dioleoyl or *sn*-1-stearoyl-2-arachidonoyl species of) phosphatidic acid, phophatidylcholine; PI, (bovine brain) phosphatidylinositol; PIP, PIP_2, PI-4-phosphate and PI-4,5-diphosphate; SUV, small unilamellar vesicles prepared by sonication.

2. MATERIALS AND METHODS

1-[3-*sn*-phosphatidyl]-D-myo-inositol (PI), 1-[3-*sn*-phosphatidyl]-D-myo-inositol-4-phosphate (PIP) and 1-[3-*sn*-phosphatidyl]-D-myo-inositol-4,5-diphosphate (PIP_2) (all from bovine brain), 1-stearoyl-2-arachidonoyl-3-*sn*-glycerophosphatidic acid (SAPA) and neomycin sulfate (95% neomycin B, 5% neomycin C) were purchased from Sigma. Isolation and purification of other lipids were as described in [8–10]. Lipid was determined on a phosphorus basis [11]. Doxorubicin was purchased from Aldrich (Belgium). Stock solutions were prepared shortly before use and were shown by HPTLC [2] to be pure. Concentrations were determined as in [6]. All other chemicals were of analytical grade (Merck, Germany). All buffers contained 100 mM NaCl, 10 mM PIPES, 5 mM EGTA, pH 7.4 (adjusted with NaOH). Monolayers were spread from solutions in $CHCl_3$ or (in case of PIP_2) $CHCl_3/CH_3OH/H_2O$, 75:25:2 (v/v). The subphase was continuously stirred; the surface pressure was recorded at room temperature with a platinum or paper (Schleicher and Schüll nr. 595) Wilhelmy plate and a Cahn 2000 electrobalance. Measurements were performed both at constant area - [12] and at constant surface pressure [13] after equilibration of the drug in interaction with the monolayer. Small lipid aggregates were prepared by sonication according to [6] resulting in SUV or (in case of pure PIP or PIP_2) micelles [14]. Doxorubicin fluorescence was recorded in an Aminco SPF 500 fluorimeter. SUV (micelles) were titrated into a continuously-stirred cuvette containing 2.5 ml buffer with 10 μM doxorubicin at 25°C. Data were corrected for light scattering (usually

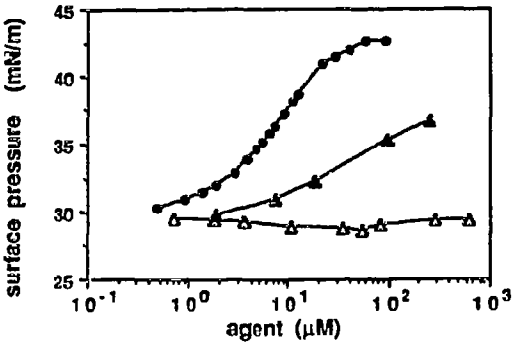

Fig. 1. Doxorubicin- or neomycin-induced increase of surface pressure in PIP_2 monolayers. The agents corresponding to the abscissa were: (●), doxorubicin; (△) neomycin; (▲), doxorubicin in the presence of 630 μM neomycin. The data were obtained after full equilibration of the drug. Equilibrium was reached within a few minutes. The subphase (5 ml) further contained: 100 mM NaCl, 10 mM PIPES, 5 mM EGTA, pH 7.4. The monolayer area was 4.9 cm^2.

negligible), drug dilution and light absorbtion (correction factor: $10^{(A_{ex}+A_{em})}$, A_{ex} and A_{em} being the absorbances at the excitation and emission wavelengths: 490 and 594 nm, respectively).

3. RESULTS AND DISCUSSION

3.1. *Penetration of doxorubicin into phosphoinositide monolayers*

At an initial surface pressure close to physiological values (30 $mN \cdot m^{-1}$ (c.f. [12,13,15]) and at constant monolayer area, doxorubicin elicited a large pressure increase in monolayers consisting of PIP_2. An example is shown in Fig. 1. These data show that the drug penetrates into the monolayer. To assess the relative strength of binding, we compared the effect of doxorubicin with that of the polyphosphoinositide-specific antibiotic neomycin [16]. No increase of surface pressure was induced by neomycin up to 630 μM (Fig. 1) showing that this hydrophilic drug did not penetrate. In agreement with the electrostatic nature of the binding of the drugs [6,16], neomycin competed efficiently with doxorubicin for binding to PIP_2 (Fig. 1). At equal concentrations of both drugs (630 μM), the effect of doxorubicin was approx. 50% smaller than in the absence of neomycin, suggesting that the affinities of both drugs for PIP_2 are comparable. Indeed the apparent affinity of doxorubicin emerging from drug penetration (Fig. 1) is comparable to the binding affinity of neomycin emerging from ref. [16].

To investigate the specificity of doxorubicin-(poly)phosphoinositide interactions, we studied in a comparative way the interactions with model systems containing PIP_2, PIP, PI, DOPA, cardiolipin (the latter two for comparison with previous studies [2-6]) and SAPA (having a similar fatty acid composition as bovine brain phosphoinositides [17]). In the experiments shown in Fig. 2, drug penetration was monitored by the drug-induced increase of the monolayer area at constant surface pressure (30 $mN \cdot m^{-1}$), in analogy to [13]. Fig. 2A shows the data of pure anionic phospholipid monolayers and Fig. 2B those of mixtures of 20 mol% anionic phospholipid with 80 mol% DOPC. Of all anionic phospholipids tested, the largest drug-induced effects were elicited with PIP and PIP_2, at least in the physiologically more relevant mixtures with DOPC (Fig. 2B). With pure DOPC, only a very small increase was obtained, in agreement with its low drug-binding capacity [6].

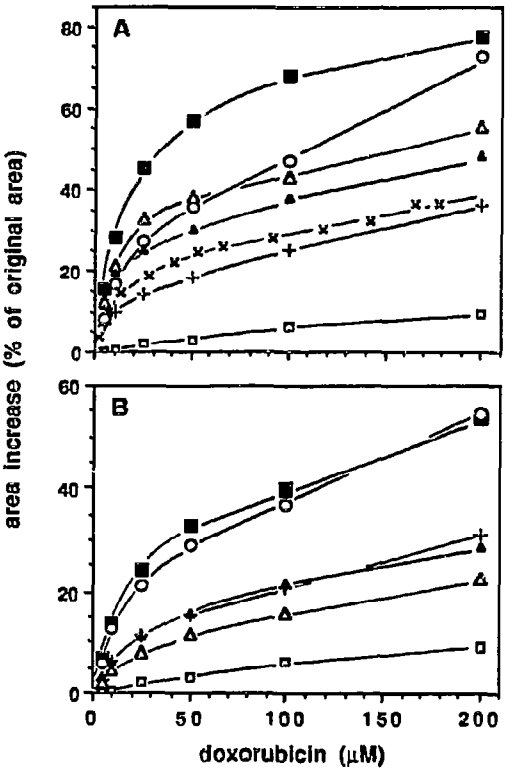

Fig. 2. Doxorubicin-induced area increase in monolayers at a constant surface pressure of 30 $mN \cdot m^{-1}$. Data were obtained after full equilibration of the drug. A, pure lipids; PIP (■), PIP_2 (○), PI (△), cardiolipin (▲), SAPA (×), DOPA (+), DOPC (□). B, mixtures of 80 mol % DOPC with 20 mol % of either: PIP (■), PIP_2 (○), DOPA (+), cardiolipin (▲), or PI (△). For comparison: pure DOPC (□). Note the different scalings in panels A and B. The subphase was 50 ml, the starting monolayer area 32 cm^2. Further conditions as in Fig. 1.

It is noteworthy that also on a molecular basis, PIP or PIP_2 admixed with DOPC elicited the largest effects. We will shortly discuss this. The initial monolayer area was always the same. Thus, the total amount of lipid present in the monolayer varied according to the inverse of its molecular area. We determined the molecular area of the pure lipids (at 30 $mN \cdot m^{-1}$): 0.94 (PIP_2, PIP), 0.75 (PI), 0.69 (PA) and 0.66 (DOPC) nm^2. From the literature [5,18], a value of 1.25 nm^2 can be obtained for cardiolipin. The amount of lipid molecules in the monolayers can thus be calculated. On the basis of these data, and those of Fig. 2A (pure lipids), the average

drug-induced area increase per lipid molecule can be calculated: 0.64 (PIP), 0.44 (PIP_2), 0.32 (PI), 0.48 (cardiolipin), 0.20 (SAPA), 0.17 (DOPA) and 0.04 (DOPC) nm^2 at 100 μM doxorubicin. In the lipid mixtures in the absence of drug, the overall area corresponded to the weighted average of the pure lipid components. The drug-induced increase of area per unit consisting of 1 anionic phospholipid plus 4 DOPC molecules (Fig. 2B) was: 1.43 (PIP), 1.33 (PIP_2), 0.54 (PI), 0.84 (cardiolipin) and 0.70 (DOPA) nm^2 at 100 μM drug. Clearly, the largest effects were obtained with PIP and PIP_2.

The area increase in mixtures of DOPC and PIP_2, PIP, PA, or cardiolipin (Fig. 2B) is larger than expected on the basis of the effects obtained with the pure lipids (Fig. 2A). The DOPC molecules that surround the putative complex between the anionic phospholipid and the drug [6] provide probably more room for penetration of the hydrophobic anthracyclinone moiety of the drug. Due to electrostatic effects, the initial degree of proton dissociation of pure PIP_2, PIP and PA is probably lower than in mixtures with DOPC. This effect might also contribute to the increased penetration of the drug in lipid mixtures containing DOPC, at low drug concentrations. At high concentrations, doxorubicin is able to displace eventually both protons from the phosphorus of DOPA [6], and it is to be expected that 3 and 5 protons can be displaced from PIP and PIP_2 respectively, provided that sterical hindrance does not occur.

Unfortunately, the overall drug-induced increase of area does not allow a precise calculation of the amount of penetrating drug, since the molecular surface of the anthracyclinone may vary from 0.1–0.7 nm^2 depending on its orientation, and since the intrinsic packing of the lipids may depend on drug incorporation.

Although PIP and PIP_2 bind more doxorubicin than the other lipids, the apparent binding affinity of the various anionic phospholipids including the polyphosphoinositides is similar. The concentration dependence of the doxorubicin-induced area increase (Fig. 2) displayed biphasic characteristics, in agreement with doxorubicin binding to large unilamellar vesicles prepared by extrusion [6]. The first phase was complete around 100 μM drug and was apparently half-saturated at 5–10 μM drug. This is in reasonable agreement with the free drug concentrations (15–35 μM) previously shown [6] to saturate 50% of all potential binding sites, including non-penetrating sites, in anionic phospholipid bilayers. The high-affinity phase was not observed with DOPC (Fig. 2) and was specific for anionic phospholipids. The second phase (at 100–200 μM drug) could represent aspecific binding [3]. On the other hand, it does not contrast with the characteristics of doxorubicin binding to anionic phospholipid bilayers, which approaches saturation only around 500 μM free drug [6]. Analysis of the electrostatic redistribution of free drug close to the membrane surface (not shown, de Wolf, unpublished results) indicates that binding saturation is caused mainly by saturation of negatively-charged binding sites (charge neutralization), rather than by depletion of free drug at the membrane surface.

In the absence of lipid, doxorubicin elicited a surface pressure of only 0.2 $mN \cdot m^{-1}$ at 20 μM, or 14–15 $mN \cdot m^{-1}$ at 250 μM (not shown). Since these values are lower than the initial surface pressures of the lipid monolayers used presently, the above-described effects are indeed due to drug-lipid interactions.

3.2. *Doxorubicin binding to phosphoinositide vesicles (self-association)*

At sufficient drug density in the membrane, self-association of doxorubicin can occur at the membrane surface of anionic phospholipids [6], resulting in quenching of its fluorescence [6]. The patterns of doxorubicin self-quenching shown in Fig. 3 demonstrate that such self-association also occurs in phosphoinositide model membranes. At high SUV or micelle concentrations, as the majority of the drug is complexed, the self-quenching is reversed due to dilution of the drug in the membranes. This indicates that the binding

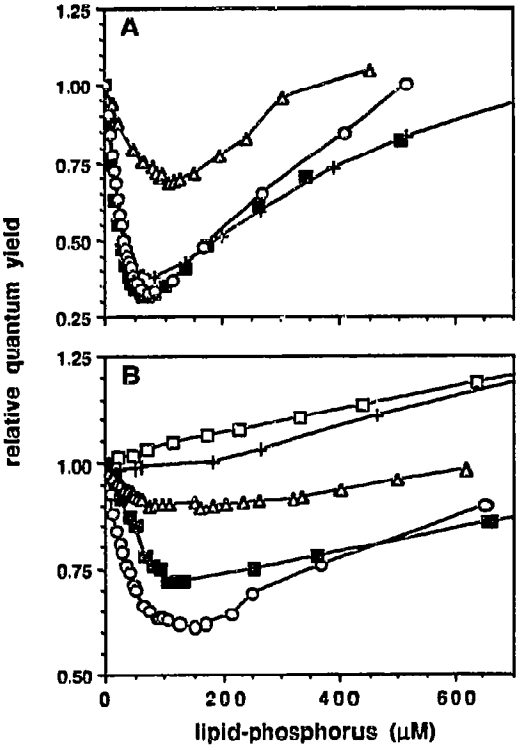

Fig. 3. Fluorescence of 10 μM doxorubicin as a function of SUV or micelle concentration (on a phosphorus basis). A, pure lipids; PI (Δ), DOPA (+), PIP_2 (○), PIP (■). B, mixtures of 75 mol % DOPC with 25 mol % of either: DOPA (+), PI (Δ), PIP (■), or PIP_2 (○). For comparison: pure DOPC (□). Excitation was at 490 nm, emission at 594 nm. The flurorescence yield, obtained after full equilibration of the drug, was normalized to the yield in the absence of lipid. Equilibration was reached within a few minutes. Further conditions as in Fig. 1.

is readily reversible and that the drug equilibrates between all SUV (micelles). In analogy to the monolayer data, the largest quenching was obtained with PIP and PIP_2. The difference with other anionic phospholipids (PI, DOPA) was especially large in the physiologically more relevant mixtures with DOPC.

In general, the extent of quenching increased with the number of potential electrostatic binding sites (on the phosphorus atoms of the lipids). It was shown that doxorubicin can displace protons from PA (pK_a 7.7) so as to disclose finally two electrostatic binding sites per phosphorus [6]. According to the pK_a of PIP and PIP_2 [14], a similar phenomenon is expected for those lipids. PI induced a larger quenching than PA or (not shown) cardiolipin, in mixtures with DOPC. As discussed above, the average doxorubicin-induced increase of area per molecule lipid was smaller in mixed PI/PC monolayers than in PA/PC or cardiolipin/PC monolayers. Possibly, a relatively larger fraction of the bound drug is self-associated at the surface of PI/PC than at the surface of PA/PC membranes. The origin of this effect is not known. In agreement with Fig. 2, Fig. 3 suggests that the apparent binding affinity of the various anionic phospholipids was similar.

We showed previously that in phospholipid membranes, doxorubicin is present in at least two distinct pools: (1) penetrating fluorescent drug, and (2) (only at sufficient drug density in the membrane, reached only in anionic phospholipids) self-associated non-fluorescent drug at the membrane surface [6]. The present data indicate that such an organization occurs also in phosphoinositide-containing membranes and that PIP and PIP_2 bind significantly more doxorubicin in either of the two membrane pools than the other anionic phospholipids. The quantitative differences between PIP_2, PIP and other anionic phospholipids are especially striking in mixtures of these lipids with DOPC and it is expected that also in the plasma membrane, doxorubicin will specifically interact with polyphosphoinositides. The physiological relevance of our observations is supported by previous findings, which show that doxorubicin can affect phosphoinositide metabolism in cancer cells [19,20] and that monoclonal antibodies specific for PIP_2 can reverse mitogenesis and proliferation, specifically of ras-, erb B- or src-transformed cells [21,22].

Acknowledgements: This research was supported by grants from the KWF-Netherlands Cancer Foundation (Project UUKC-88-06). Hermen van Dalen and Martijn Koorengevel are acknowledged for synthesis and purification of DOPC, DOPA and cardiolipin, Albrecht Moritz and Dorus Gadella for kindly providing some PIP_2 and neomycin, respectively.

REFERENCES

[1] Capranico, G., Zunino, F., Kohn, K.W. and Pommier, Y. (1990) Biochemistry 27, 562–569.
[2] Nicolay, K., Timmers, R.J.M., Spoelstra, E., van der Neut, R., Fok, J.J., Huigen, Y.M., Verkleij, A.J. and de Kruijff, B. (1984) Biochim. Biophys. Acta. 778, 359–371.
[3] Henry, N., Fantine, E.O., Bolard, J. and Garnier-Suillerot (1985) Biochemistry 24, 7085–7092.
[4] Goormaghtigh, E. and Ruysschaert, J.M. (1984) Biochim. Biophys. Acta 779, 271–288.
[5] Nicolay, K., Sautereau, A.M., Tocanne, J.F., Brasseur, R., Huart, P., Ruysschaert, J.M. and de Kruijff, B. (1988) Biochim. Biophys. Acta 940, 197–208.
[6] De Wolf, F.A., Maliepaard, M., van Dorsten, F., Berghuis, I., Nicolay, K. and de Kruijff, B. (1991) Biochim. Biophys. Acta 1096, 67–80.
[7] Berridge, M.J. (1987) Annu. Rev. Biochem. 56, 159–193.
[8] Van Deenen, L.L.M. and de Haas, G.H. (1964) Adv. Lip. Res. 2, 168–229.
[9] Comfurius, P. and Zwaal, R.F.A. (1977) Biochim. Biophys. Acta 218, 102–111.
[10] Smaal, E.B., Romijn, D., Geurts van Kessel, W.S.M., de Kruijff, B. and de Gier, J. (1985) J. Lip. Res. 26, 633–637.
[11] Rouser, G., Fleischer, S. and Yamamoto, A. (1970) Lipids 5, 494–496.
[12] Pilon, M., Jordi, W., de Kruijff, B. and Demel, R.A. (1987) Biochim. Biophys. Acta 902, 207–216.
[13] Seelig, A. (1987) Biochim. Biophys. Acta 899, 196–204.
[14] Van Paridon, P.A., de Kruijff, B., Ouwerkerk, R. and Wirtz, K.W.A. (1986) Biochim. Biophys. Acta 877, 216–219.
[15] Demel, R.A., Geurts van Kessel, W.S.M., Zwaal, R.F.A., Roelofsen, B. and van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 97–107.
[16] Schacht, J. (1976) J. Neurochem. 27, 1119–1124.
[17] White, D.A. (1973) in: Form and Function of Phospholipids (Ansell, G.B., Hawthorne T.N. and Dawson, R.M.C., eds.) BBA Library vol. 4, pp. 441–482, Elsevier, Amsterdam.
[18] Shah, D.O. and Schulman, J.H. (1965) J. Lip. Res. 6, 341–349.
[19] Thompson, M.G., Chawala, S.B. and Hickman, J.A. (1987) Cancer Res. 47, 2799–2803.
[20] Lanzi, C., Gambetta, R.A., Perego, P., Banfi, P., Franzi, A., Guazzoni, L. and Zunino, F. (1991) Int. J. Cancer 47, 136–142.
[21] Matuoka, K., Fukami, K., Nakanishi, O., Kawai, S. and Takenawa, T. (1988) Science 239, 640–643.
[22] Takenawa, T. and Fukami, K. (1989) Clin. Chim. Acta 185, 309–316.