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Calmodulin and alpha tocopherol as additional binding sites for doxorubicin

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Summary. The hydrophobic probes anthroylcholine (9AC), 8-anilino-1-napthalene sulfonate (ANSE), and 2-P-toluidinyl naphthalene 6-sulfonate (TNS) increased calcium-calmodulin (Ca^{2+}-CaM) fluorescence. This fluorescence was decreased by doxorubicin (DXR) in a dose-dependent fashion. The Ca^{2+} ion was an absolute requirement for the observed effects of DXR. DXR bound to the Ca^{2+}-CaM complex ($K_d = 4.2 \times 10^{-5}$ *M*, $B_{max} = 1.8$) and to alpha tocopherol. The binding of untransformed (native) DXR to CaM was a reversible process. These data support a previous finding that DXR inhibits stimulation of calmodulin-deficient PDE (a CaM target enzyme) using either the Ca^{2+} CaM complex or alpha tocopherol by interacting with these agents, and suggest that other target enzymes for CaM may be similarly affected.

Introduction

Doxorubicin (DXR) is currently one of the most useful antineoplastic agents. From the time of its discovery until recently, the mechanism of its antineoplastic action has been ascribed to an interaction with DNA resulting in inhibition of DNA as well as RNA and protein syntheses [8, 9]. This DNA-receptor hypothesis is generally accepted because DXR binds to the DNA molecule with a high affinity by intercalation [3], and cytoflourescent studies have provided evidence that the drug accumulates in the nuclei of treated cells [16].

Evidence has accumulated that raises doubts as to whether DNA interaction is the only drug action responsible for the cytotoxic effects of DXR. Examples of such evidence include: (a) a questionable relationship among anthracyclines between their ability to inhibit DNA synthesis and cytotoxicity [7]; (b) an observation that *N*-trifluoroacetyl adriamycin-14-valerate (AD32), a derivative of DXR, possesses antitumor activity but does not significantly bind to DNA or locate in the nucleus of treated cells [12, 25]; and (c) recent evidence that DXR can be actively cytotoxic without entering the cell [28].

Since DXR and certain analogues can be cytotoxic solely by interacting with the cell membrane [24, 28], sites of this interaction and the physiological processes interrupted demand further investigation. We have reported that anthracyclines inhibited CaM and alpha tocopherol stimulated phosphodiesterase activity (PDE) by interacting with CaM and alpha tocopherol, respectively [23]. Since CaM and lipids are components of mammalian membranes, it seemed appropriate to study the binding of DXR to CaM and alpha tocopherol.

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Materials and methods

Chemicals. Doxorubicin hydrochloride and alpha tocopherol succinate were obtained from Drug Synthesis and Chemistry Branch, National Cancer Institute, NIH, Bethesda, MD. The 8-anilino-1-naphthalene sulfonic acid hemi-magnesium salt (ANS) practical grade, 2-*p*-toluidinyl naphthalene-6 sulfonate potassium salt (TNS) and 9-anthroylcholine (9AC) were obtained from Sigma Chemical Company, St. Louis, MO. Other chemicals, as reported previously [30], namely 2-mercaptoethanol, Trizma (Tris-HCl buffer, pH 7.4), MES [2(*N*-morpholino) ethane sulfonic acid], EGTA [ethylene glycol-bis(B-aminoethyl ether)] *N-N*^{1}-tetraacetic acid were also purchased from Sigma Chemical Company, St. Louis, Mo.

Preparation of calmodulin-deficient PDE. Calmodulin-deficient PDE was prepared by the method of Klee and Krinks [14] with some modifications, using 10 m*M* 2(*N*-morpholino)ethane sulfonic acid (MES) buffer, pH 6.7 containing 1 m*M* EGTA [ethylene glycol-bis(B-amino ethyl ether) *N,N'*-tetraacetic acid], 1 m*M* $MgCl_2$, and 5 m*M* 2-mercaptoethanol.

Purification of calmodulin. Calmodulin was purified from bovine brain by affinity chromatography over a fluphenazine-Sepharose column according to the method of Charbonneau and Cormier [5]. SDS (sodium dodecyl sulfate) polyacrylamide gel electrophoresis was used to confirm the purity of the protein. Aliquots were frozen at $-20°$ and the protein retained activity for more than 9 months.

Binding measurements. The calmodulin and alpha tocopherol binding of doxorubicin was measured by spectrofluorometry. DXR is strongly fluorescent, and the method for determining the fraction of antibiotic molecule bound was based on the fact that the fluorescence of the antibiotics is quenched when bound to calmodulin or alpha tocopherol, as with binding to DNA. The binding parameters of DXR to calmodulin were determined spectrofluorometrically with an Aminco-Boman spectrofluorometer using

5-cm light path cells. Each sample was contained in a test tube and consisted of 50 m*M* Tris-HCl, varying concentrations of DXR with or without 6 nmol calmodulin, 1 m*M* calcium or 1 m*M* EGTA, and 1 m*M* magnesium. Each sample was preincubated for 30 min and subsequently incubated for 2.5 h after the addition of TNS, ANS, or 9AC. Samples in which no probe was included were incubated for 15 min without preincubation. The fluorescence (% transmission) was measured at 380 nm excitation and emission wavelengths ranging from 400 to 620 nm, and at a meter multiplier of 0.001.

In the alpha tocopherol binding studies, each sample consisted of 1 m*M* calcium, 1 m*M* magnesium, and varying concentrations of alpha tocopherol, but the concentration of DXR (50 m*M*) was constant. The fluorescent probe used was 9AC. The fluorescence was measured at excitation wavelength of 470 nm and emission wavelengths of 535–555 nm and at a meter multiplier of 0.03 in all studies; the total volume of the reactions mixture was 1.6 ml, and incubations were carried out at 25 °C. All samples were shaken throughout the incubations.

The absorbance data were used to calculate the Scatchard plot according to the method employed by Muller and Crothers [20]. The expression used $r/m = K_{app}(B_{app}{}^{-r})$ is appropriate to the plot of binding isotherms where 'r' is the ratio of bound DXR to total CaM, 'M' is the concentration of free DXR, K_{app} is the apparent binding constant, and B_{app} is the apparent number of binding sites. All experiments were carried out in the dark.

PDE assay and reversibility of DXR binding to CaM. PDE activity was measured by the method of Thompson and Appleman [27] as modified by Boudreau and Drummond [2]. The incubation mixture contained 1 μ*M* [^{3}H]cyclic AMP, 1 m*M* $MgCl_2$, 50 m*M* Tris-HCl buffer, pH 7.4, and 2.4 n*M* CaM, 0.1 m*M* $CaCl_2$ or 0.1 m*M* EGTA in a final volume of 250 μl.

All assays were performed in either triplicate or duplicate under conditions where the rate was linear with the time of incubation and the concentration of the enzyme protein. Blanks were run concurrently with the test samples for substrate blank corrections. Units of phosphodiesterase activity were picomoles/nanomoles of cyclic AMP hydrolyzed per milligram of protein per minute.

The reversibility of DXR binding to CaM is based on the ability of drug-treated CaM to stimulate Ca^{2+}-CaM-dependent PDE activity. Essentially, the experiment consists of three parts, viz. (a) binding of the drug to CaM; (b) separation of CaM from the drug by means of dialysis; and (c) carrying out the PDE assay with recovered CaM as described above. The binding of the drug to CaM was carried out by adding the drug to the PDE assay mixture. The mixture consisted of 50 m*M* Tris-HCl, pH 7.4, 0.1 m*M* Ca^{2+}, 1 μ*M* cAMP, 29.4 n*M* CaM, and different concentrations of DXR in a total volume of 1 ml. The control did not contain the drug. After 15 min preincubation, the reaction was started by addition of the PDE enzyme. The mixture was incubated for 30 min, boiled for 2 min to stop the reaction, and then cooled in an ice bath. The control and drug-treated groups were dialyzed overnight (24 h) against 10 m*M* MES buffer, pH 7.5, containing 1 m*M* Mg^{2+} to remove cAMP 5'AMP and the drug (the buffer was changed twice during dialysis). After dialysis, the mixture was centrifuged at 3000 RPM for 10 min to remove denatured proteins. The supernatant was collected and used as the source of CaM. The PDE assay was carried out as described above.

Results

In the presence of Ca^{2+} ions, a hydrophobic probe (ANS) induced a relatively high fluorescence when bound to CaM. This increase in fluorescent intensity is illustrated in Fig. 1 as the control. When DXR was added to the medium containing Ca^{2+}-CaM-probe complex, the relative fluorescence was reduced. The reduction in the fluorescence

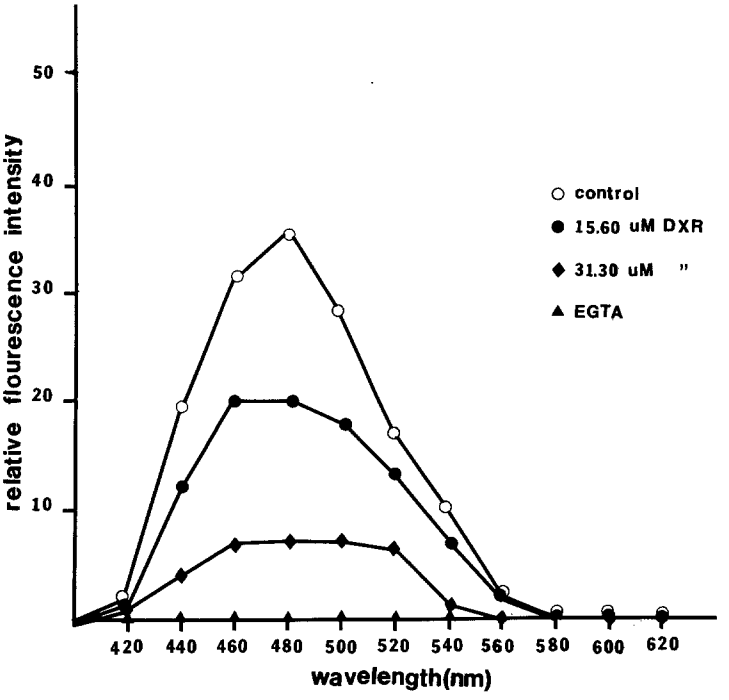

Fig. 1. Effect of doxorubicin *(DXR)* on 8-anilino-1-naphthalene sulfonic acid (ANS)-induced Ca^{2+}-CaM fluorescence. CaM (6 n*M*) was incubated with 1 m*M* Ca^{2+}, 1 m*M* Mg^{2+}, 50 m*M* Tris-HCl, different concentrations of doxorubicin as indicated, and 12 μ*M* ANS in a total volume of 1.6 ml. The EGTA incubation mixtures contained all reactants except Ca^{2+} and CaM. The samples were preincubated for 30 min and subsequently incubated for 2.5 h after addition of the probe. Fluorescence (% transmission) was measured with excitation at 380 n*M* and with emission over the range of 400–620 n*M* wavelengths

was directly proportional to the dose of the drug added. For example, 15.60 μ*M* of DXR decreased the relative fluorescence by 43%, whereas 31.30 μ*M* caused an 86% reduction of the fluorescence. This drug-induced reduction in fluorescent intensity was Ca^{2+}-dependent, since no fluorescence was observed in the presence of EGTA. Similar results were obtained with other hydrophobic probes, viz. 9AC and TNS, as shown in Figs. 2 and 3, respectively. These findings suggest that the binding site of DXR on CaM is a hydrophobic site exposed by binding of Ca^{2+} ions [17]. If this were the case, DXR and the hydrophobic probes might be expected to share common binding sites. To test this possibility, the reduction of hydrophobic probe-induced CaM fluorescence by DXR was investigated in the presence of increasing concentrations of a probe.

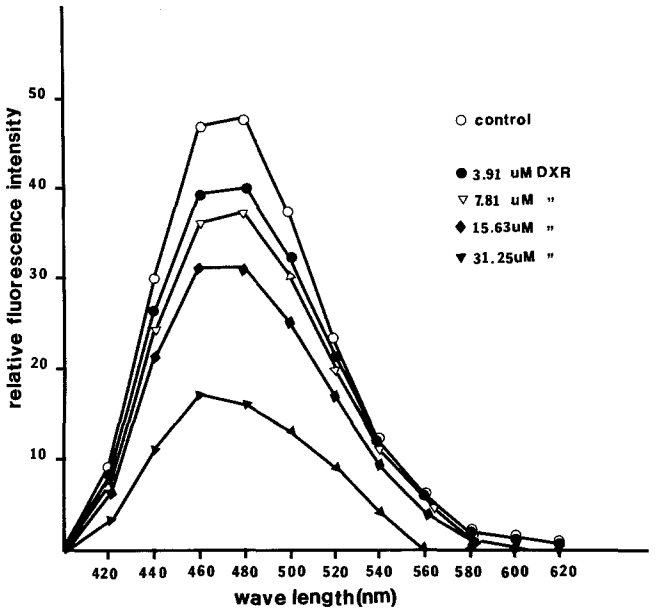

Fig. 2. Effect of doxorubicin *(DXR)* on anthroylcholine *(9AC)*-induced Ca^{2+}-CaM fluorescence. The incubation mixtures and the method are the same as described for ANS in Fig. 1, except that 10 μM 9AC and additional concentrations of the drug were used

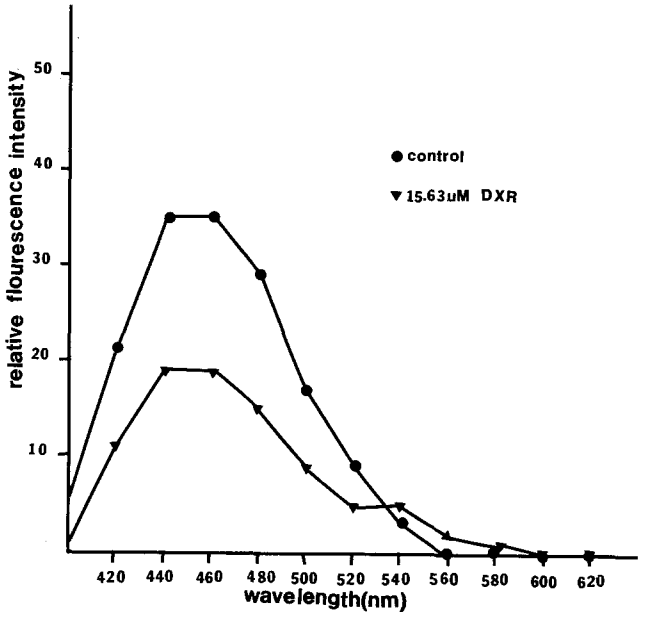

Fig. 3. Effect of doxorubicin *(DXR)* on 2-*p*-toluidinyl naphthalene-6-sulfonic acid *(TNS)*-induced Ca^{2+}-CaM fluorescence. The incubation mixtures and the method are the same as described for ANS in Fig. 1, except that 15 μM TNS and 15.63 μM doxorubicin were used.

The results in Table 1 show that increasing the concentration of ANS from 0.75 μM to 6.00 μM did not alter the binding of DXR to CaM. Similar results were obtained with an increase in the concentration of 9AC from 3.85 μM to 12.5 μM, as also shown in Table 1. The reason for the increase in percentage bound at the 12-μM concentration of ANS was not investigated.

Table 1. Effect of increasing the concentration of 8-anilino-1-naphthalene sulfonic acid (ANS) and 9-anthroylcholine (9AC) on doxorubicin-induced reduction of CaM fluorescence

Probe (dye)	Concentration of probe (μM)	% Bound drug
ANS	0.75	52.0
	1.50	51.2
	3.00	48.9
	6.00	51.7
	12.00	75.0
9AC	3.85	61.1
	5.00	68.0
	6.25	60.0
	8.33	58.8
	12.50	62.7

Reactants, 6 nmol CaM, 1 mM Ca^{2+}, 1 mM Mg^{2+} and 22.3 μM doxorubicin, were preincubated for 30 min and subsequently incubated for 2 h at 25°C after the addition of different concentrations of the probe. The fluorescence of the samples was measured at 380 excitation and over the range of 400 – 620 emission wavelengths. The percentage of drug bound to CaM is assumed to be proportional to the drug-induced reduction in CaM-probe fluorescence

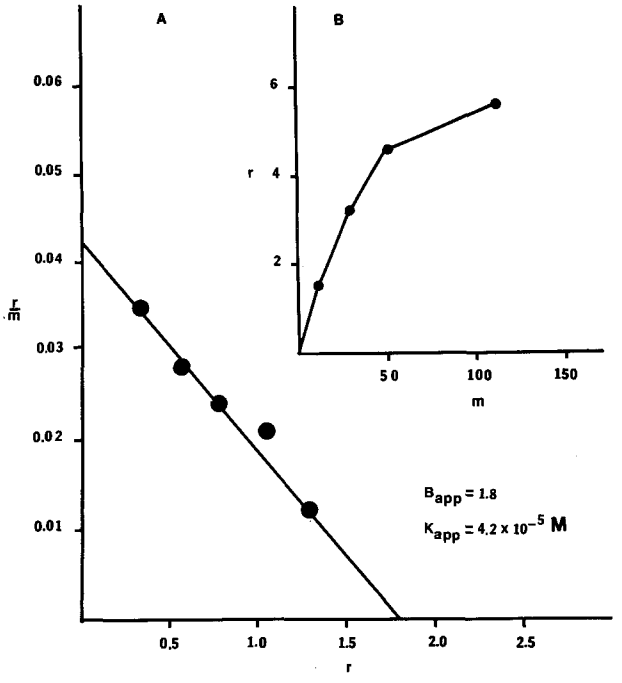

Fig. 4, A, B. Scatchard plot **(A)** and bounds vs free doxorubicin curve **(B)** for binding of doxorubicin to CaM. Different concentrations of doxorubicin were incubated with 1 mM Mg^{2+}, 1 mM Ca^{2+}, 6 nmol CaM, and 50 mM Tris-HCl in a total volume of 1.6 ml for 15 min. Fluorescence (% transmission) was measured at 470 nM excitation and over the range of 535–555 nM emission wavelengths. *r*, ratio of bound doxorubicin to total CaM; *M* concentration of free doxorubicin

Figure 4 illustrates the Scatchard plot of the binding of DXR to CaM. The apparent binding constant (K_{app}) was 4.2×10^{-5} *M*, whereas 1.8 molecules of DXR bound to one molecule of CaM.

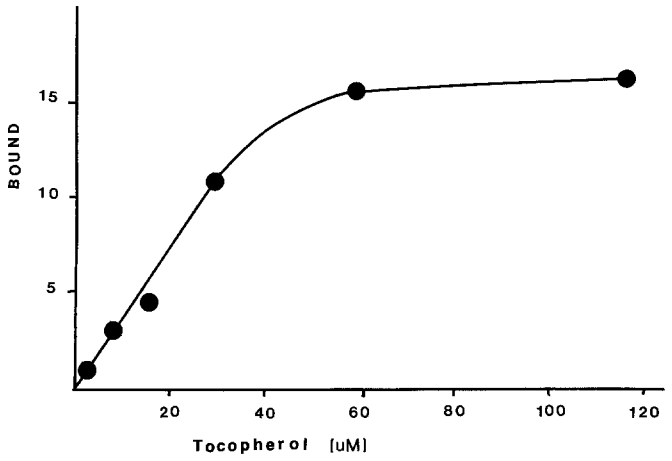

Fig. 5. Binding of doxorubicin to alpha tocopherol. Different concentrations of alpha tocopherol succinate were incubated with 1 mM Mg^{2+}, 50 μM doxorubicin, 50 mM Tris HCl in a total volume of 1.6 ml for 15 minutes. Fluorescence (% transmission) was measured with excitation at 470 nM and with emission at 535-555 nM wavelengths

Table 2. Reversible binding of doxorubicin (DXR) to CaM

Treatment	CaM Concentration (n*M*) after treatment	PDE activity (Pmol/nmol/ mg protein/min
Control (only CaM)	2.4	2.2
	4.7	3.7
	7.1	6.0
	9.4	7.3
	11.8	8.7
5×10^{-4} *M* DXR + CaM	2.4	2.0
	4.7	2.8
	7.1	5.0
	9.4	6.1
	11.8	6.9
2.5×10^{-4} *M* DXR + CaM	2.4	2.1
	4.7	2.9
	7.1	5.7
	9.4	6.6
	11.8	7.6
5×10^{-5} *M* DXR + CaM	2.4	2.2
	4.7	4.5
	7.1	6.3
	9.4	7.5
	11.8	8.6

Different concentrations of doxorubicin were incubated with 50 m*M* Tris – HCl pH 7.4, 0.1 m*M* Ca^{2+}, 1 μ*M* cAMP, and 29.4 n*M* CaM in a total volume of 1 ml, white controls contained all the reactants except the drugs. Both the drug-treated and the drug-free CaM were preincubated for 15 min and subsequently incubated for 30 min, thereafter boiled and cooled. CaM was recovered by dialysis and centrifugation. The recovered CaM was used to assay for PDE activity

DXR also binds to alpha tocopherol and this binding seemed saturable as shown in Fig. 5. Approximately 8 μ*M* DXR binds to 21 μ*M* alpha tocopherol at half maximum binding.

The binding of DXR to CaM can be considered reversible only when the drug-treated CaM is capable of performing its function to the same degree as the untreated CaM. Table 2 shows that CaM treated with 5×10^{-4} *M* DXR, 2.5×10^{-4} *M* DXR and 5×10^{-5} *M* DXR stimulated PDE activity in a dose-dependent fashion similar to the stimulation of PDE activity by drug-free CaM. However, the CaM that was treated with 2.5×10^{-4} *M* DXR and 5×10^{-4} *M* DXR possessed comparatively less PDE-stimulatory capacity than the drug-free protein activator, whereas the 5×10^{-5} *M* DXR-treated CaM has the same PDE-stimulatory potency as the untreated protein.

Discussion

The data demonstrate that 1.8 mol DXR bind to 1 mol CaM with a binding constant of 4.2×10^{-5} *M*. The binding of doxorubicin to CaM was observed only in the presence of Ca^{2+} ions and not in the presence of EGTA, suggesting that DXR binds to the hydrophobic sites which are exposed consequent to the binding of Ca^{2+} ions to CAM [17]. Further, the hydrophobic probes and DXR bind to CaM at hydrophobic sites. However, increasing the concentration of the hydrophobic probes did not alter the percentage of the DXR bound to CaM. This result may be explained by: (a) the affinity of the DXR may be much greater than that of the probes for CaM at the hydrophobic sites and hence the inability of the probe to displace DXR; (b) the number of binding sites for the probes on CaM may be greater than those for DXR so that increasing concentrations of the probe does not have any significant effect on the binding of DXR to CaM; or (c) the DXR and the probes may have different binding sites in the hydrophobic domain of CaM. Explantions (a) and (b) seem valid, since it has been reported that Ca^{2+} CaM complex has four to six binding sites for 9AC with a K_d of 440 μ*M* and two to three binding sites for ANS with a K_d of 490 μ*M* [17].

The binding of the DXR to alpha tocopherol showed a saturable phenomenon, indicating that the drug also forms a stable alpha tocopherol-drug complex. DXR forms molecular associations with a variety of aromatic and planar molecules and with other DXR molecules in aqueous solutions [6]. These results with alpha tocopherol are consistent with the reports that DXR binds to phospholipids, proteins, and other hydrophobic components [10, 19, 29]. Further, we have observed that when DXR and CaM were incubated in a medium of 100 m*M* NaCl, the binding of DXR to CaM but not alpha tocopherol, was greatly reduced (data not shown). This last finding suggests that electrostatic interaction between the positively charged amino group of the sugar residue in DXR and the negatively charged CaM may be important in the binding of the drug with CaM.

The binding of DXR to CaM is reversible since the 5×10^{-5} *M* DXR-treated CaM was capable of stimulating CaM-dependent PDE activity to the same degree as the untreated CaM. However, we noticed that CaM treated with a 2.5×10^{-4} *M* or 5×10^{-4} *M* DXR were less active in stimulating PDE activity than the untreated CaM protein. These latter concentrations of DXR greatly exceed con-

centrations achievable in vivo. Since the experiments were carried out in dark, it is assumed that the DXR used in this study is in its native form. Trifluoperazine also binds to CaM reversibly, but when it is transformed into reactive-free radical forms the binding becomes irreversible [31]. Potentially, DXR may be similarly transformed. The reported competitive inhibition of Ca^{2+} CaM and alpha tocopherol stimulation of PDE by DXR [22, 23] is apparently brought about by reversible binding of DXR with CaM or alpha tocopherol. In tissues such as testis and heart [15, 26], and in certain tumor cell lines [15, 18] which have high CaM levels, binding of DXR to CaM may facilitate the accumulation of DXR. However, the uptake and extent of binding in cells are controlled by physiological factors such as regional blood flow and active efflux mechanisms [11]. Indeed, the adverse effects on the myocardium are well known, and the testis in the rat seems to accumulate DXR [22]; or indirectly, an IV bolus of 1 mg/kg dose once weekly for 9 weeks results in cumulative toxicity to the testis. Hence, tissues rich in CaM may be susceptible to the effects of DXR, and if active efflux of DXR from cells is lacking the tissue may exhibit an increased susceptibility.

CaM is an important component of membranes, especially sarcolemma and the sarcoplasm reticulum where it regulates ion fluxes [4, 13]. The binding of DXR and the oxidative destruction of erythrocyte ghost membranes by DXR-free radicals has also been reported [21]. In addition, DXR binds to cardiolipin in mitochondrial membranes and spectrin in the erythrocyte membrane (20,1 and 2). Similarly *d*-alpha tocopherol has been reported to bind specifically to intact human erythrocyte membranes [32]. Although the exact physiological function of *d*-alpha tocopherol is not known, it is thought to function as an antioxidant, to influence membrane-bound enzymes, and to protect erythocytes and leukocytes from oxidative membrane damage associated with hemolysis or decreased phagocytosis [32]. The result of this study presents CaM and alpha tocopherol as relatively high-affinity binding sites for DXR. Further, these data support our previous finding that DXR inhibits the stimulation of PDE using either the Ca^{2+}-CaM complex or alpha tocopherol by interacting with these agents, and suggest that other target enzymes for CaM may be similarly affected.

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