Interaction of Cu(II) and Cu(II)–anthracycline complexes with protein kinase C. Spectromagnetic assessment of the inhibitory effect

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Abstract

Anthracycline antitumor drugs weakly inhibit protein kinase C (PKC) activity; this effect is enhanced by coordination with transition metal ions. The present study shows that copper(II) by itself inhibits PKC activity; when Cu(II) is present as the anthracycline–Cu(II) complex [Cu(EpiDXR)$_2$], a significant enhancement is observed as compared to the inhibitory effects of either metal or drug alone. Both regulatory and catalytic sites of PKC are involved in this effect. Electron paramagnetic resonance (EPR) studies indicate an interaction between PKC and Cu(II). It is suggested that Cu(II) is responsible for the inhibitory action of Cu(II)–anthracycline complexes on PKC; anthracycline molecules potentiate this effect, possibly by stabilizing Cu(II) and increasing its availability at enzyme target sites.

Introduction

In the last ten years several studies have been published indicating that some of the effects produced by anthracycline antitumor agents could be mediated by coordination of transition metal ions. A possible shuttle role was proposed for the metal in the electron transfer processes between anthracycline and molecular oxygen, leading to free radical production and subsequent damage to DNA and other macromolecular targets [1–3]. The behavior of copper(II) and iron(III) anthracycline derivatives was described in detail [4, 5], due to their easy detection and to the ability to assess anthracycline coordination to the metal, particularly in the case of Cu(II), by electron paramagnetic resonance (EPR) spectroscopy [6–9].

Recent trends in the development of new anticancer agents indicate a possible therapeutic goal, as an alternative to DNA damage, in the inhibition of specific cell functions, such as signal transduction pathways triggered by growth factor receptors or by tumor promoter molecules. Chelation of transition metal ions by anthracyclines, which appears to enhance the inhibitory action exerted by these drugs on protein kinase C (PKC) activity [10], has been investigated as an example of this novel type of approach.

The calcium-activated, phospholipid dependent PKC is a family of closely related isoenzymes involved in many cell functions, such as cell growth and differentiation, hormone secretion, transduction of extracellular signals [reviews, 11–14]. It has been reported that metal ions [15] and drugs, such as doxorubicin (DXR), 4′-epidoxorubicin (EpiDXR), chlorpromazine, trifluoperazine and tamoxifen [16], independently exert an inhibitory action on PKC activity, possibly by interacting with the regulatory domain of the enzyme. It has also been shown that a transition metal complex of DXR, the DXR–Fe(III) complex [Fe(DXR)$_3$], develops a potent inhibition by interacting with both the regulatory and the catalytic site of PKC [10]. However, no conclusive evidence has been provided as to the existence of an interaction of the enzyme with the metal and the drug–metal complex; therefore, the molecular mechanism of the inhibitory effect remains unclear.

In order to better understand the role of transition metal ions, as such or as anthracycline complexes, in the inhibition of PKC, we studied the effect produced on PKC by Cu(II) and its EpiDXR derivative [Cu(EpiDXR)$_2$]. EpiDXR (Scheme 1) was chosen because the structure and chemistry of its Cu(II) complexes have been extensively documented by this group [6–9].

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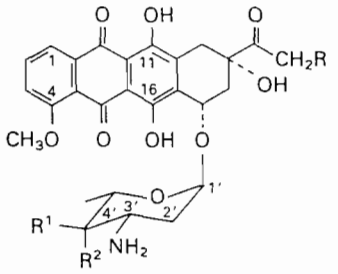

R = OH; R^1 = H; R^2 = OH doxorubicin (1)

R = OH; R^1 = OH; R^2 = H 4′-epidoxorubicin (2)

Scheme 1.

Moreover the metal interaction with neighboring groups can be monitored by EPR spectroscopy. DXR was not used in this study because its Cu(II) complexes exhibit a polymeric structure, which would considerably complicate their detection by EPR spectroscopy.

Methods

4′-Epidoxorubicin (EpiDXR) was supplied by Farmitalia–C. Erba, Milano. The 4′-epidoxorubicin–Cu(II) complex [Cu(EpiDXR)$_2$] was prepared by mixing aqueous solutions of EpiDXR and of $CuCl_2 \cdot 2H_2O$ (analytical grade) in a 2:1 molar ratio and back-titrating to pH 7.0.

PKC purification

PKC was purified from rat brain according to Kitano *et al.* [17]. Briefly, 3 g of rat brains were homogenized in Tris 20 mM (pH 7.5) containing sucrose 0.25 M, EGTA 10 mM, EDTA 2 mM, phenylmethyl sulfonyl fluoride 1 mM and 20 μg/ml leupeptin. The supernatant of a 60 min centrifugation at 100 000 g was applied to a DEAE cellulose column previously equilibrated with Tris 20 mM (pH 7.5) containing EDTA 0.5 mM, EGTA 0.5 mM and 2-mercaptoethanol 10 mM (buffer A). The enzyme was eluted with 90 mM NaCl in buffer A. The enzyme solution was directly applied to a threonine-Sepharose 4b column equilibrated with buffer A. The enzyme was eluted by a linear gradient of NaCl (0.6 to 1.0 M NaCl) in buffer A. The eluate was subsequently applied to a phenyl Sepharose CL-4B column equilibrated with 1 M NaCl in buffer A. The column was washed with the same buffer. Then, the enzyme was eluted from the column by linearly decreasing the concentration of NaCl from 1.0 to 0 M. This procedure provides electrophoretically pure enzyme at an approximate concentration of 0.5 μM in buffer A.

PKC enzymatic assay

Fractions collected from the phenyl Sepharose column were tested for PKC activity by measuring the incorporation of ^{32}P from [g-^{32}P]ATP (Amersham, Italy, 2 mCi/ml, 3000 Ci/mmol) into type III Histone. The assay reaction solution (ARS) contained 50 mM Tris (pH 7.5), 12 mM $MgCl_2$, 20 μM [g-^{32}P]ATP (10^6 cpm/nmol), type III Histone (300 μg/ml), phosphatidylserine (50 μg/ml), diolein (5 μg/ml), $CaCl_2$ (100 μM); PKC was added in buffer A to a final concentration of 0.1 μM. After incubation for 15 min at room temperature the reaction was stopped by adding 20% trichloroacetic acid. The contents of the reaction test tubes were spotted onto P81 paper (Whatman), the paper was washed in 0.5% orthophosphoric acid and dried and radioactivity was quantitated by standard scintillation techniques. Baseline kinase activity was measured in the absence of calcium, phosphatidylserine and diacylglycerol.

PKC activity was tested as drug concentration inhibiting 50% of the enzyme activity (IC_{50}), in the presence of different concentrations of EpiDXR, Cu(II) and [Cu(EpiDXR)$_2$], ranging from 5.0 to 100.0 μM for Cu(II) and the complex and from 10.0 to 200.0 μM for EpiDXR, which was present in a 2:1 molar ratio to Cu(II).

EPR studies

EPR spectra for Cu(II) and [Cu(EpiDXR)$_2$] were recorded on a separate set of samples incubated in the same assay reaction solution described for PKC assays, in which [g-^{32}P]ATP had been replaced by unlabeled ATP. Where appropriate, Cu(II) or [Cu(EpiDXR)$_2$] were added to ARS to a final concentration of 200 μM (expressed as copper content). When PKC was omitted from the samples, an equal volume of buffer A was added to ARS. 10% ethylene glycol was added in order to have glassing solutions for EPR measurements at 123 K. EPR spectra of frozen samples were recorded by a Varian E-109 spectrometer, working at X-frequency band and equipped with a variable temperature apparatus. The experimental conditions adopted are indicated in the legend of Fig. 3.

Results and discussion

In the present investigation we studied the role of copper(II) and of its complexes with anthracycline antitumor agents in inhibiting PKC activity. The rationale for the choice of EpiDXR, instead of the parent compound DXR, lies in the well defined structural properties and spectromagnetic characterization of its Cu(II) complexes and particularly of the complex obtained at a drug:Cu(II) molar ratio of 2:1. In contrast, the Cu(II) complex obtained with DXR at the same drug:Cu(II) molar ratio yields a poorly resolved EPR spectrum, due to its polymeric structure [11–13].

Figure 1 shows that Cu(II) alone inhibited PKC activity in a dose-dependent fashion in the concentration range 5.0–100 μM, with a IC_{50} of approximately 100 μM. On the other hand, EpiDXR alone brought about a moderate inhibition of PKC activity, which reached a plateau at approximately 85% of control for drug concentrations greater than 10 μM. The degree of inhibition observed with $[Cu(EpiDXR)_2]$ was found to be significantly higher ($IC_{50} = 9.2\ \mu M$) than the inhibition produced by either agent by itself and yielded a synergistic effect. The inhibition by $[Cu(EpiDXR)_2]$ is comparable with that obtained by Hannun *et al.* with $[Fe(DXR)_3]$ [10]. This behavior differs from that reported by the same authors for Cu(II)–DXR complexes; however, the authors did not provide details on the stoichiometry of the complexes and on the experimental conditions adopted and measures of the inhibitory action of Cu(II)–DXR derivatives were not reported [10].

The results of experiments performed to study the site of action of Cu and of $[Cu(EpiDXR)_2]$ in the PKC molecule are reported in Fig. 2. Lineweaver–Burk analysis of the data obtained for $[Cu(EpiDXR)_2]$ in the presence of different Ca^{2+} concentrations indicates a non-competitive type of interaction with the Ca^{2+}-binding site of the regulatory domain (Fig. 2(a)). As pointed out by Hannun *et al.* [10], double reciprocal analysis of the interaction with the phosphatidylserine (PS)-binding site is precluded by the highly cooperative effects of PS on PKC. Therefore, direct plots have been reported in this case, showing that the interaction with the PS-binding site is also of a non-competitive type (Fig. 2(b)). This interaction of Cu(II) and Cu(II)–anthracycline derivatives with the regulatory domain could reasonably be predicted, based on the presence of binding sites for anthracyclines [15] and

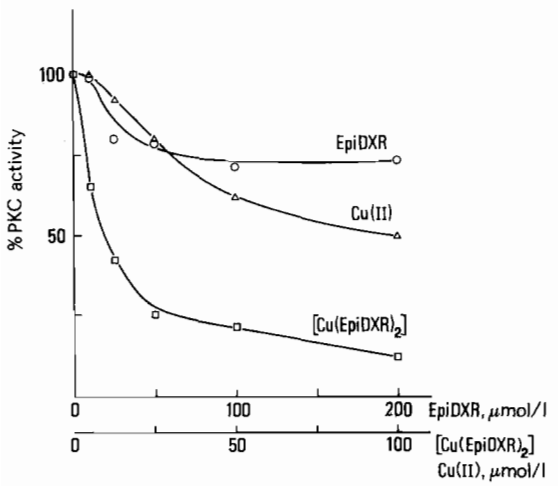

Fig. 1. PKC inhibition by EpiDXR, Cu and $[Cu(EpiDXR)_2]$. The concentration of $[Cu(EpiDXR)_2]$ is calculated on the basis of the Cu(II) content (lower scale).

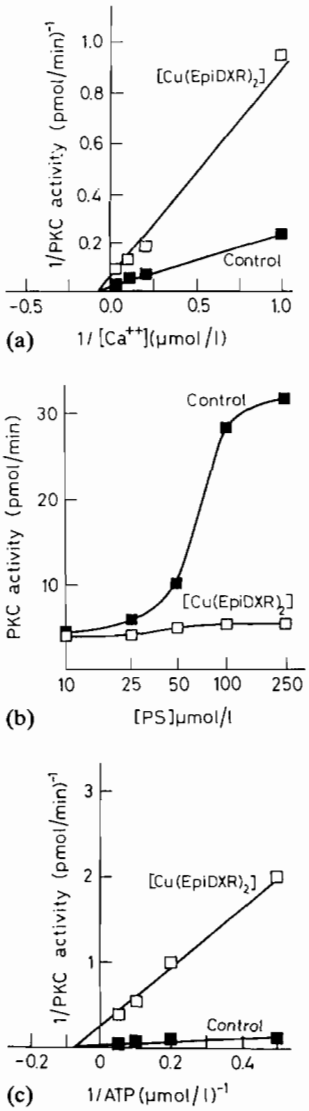

Fig. 2. Interaction of $[Cu(EpiDXR)_2]$ with calcium, phosphatidylserine, ATP and its effect on PKC activity. A 50 μM concentration (calculated on the basis of the Cu(II) content) of $[Cu(EpiDXR)_2]$ was used. (a) Double reciprocal plots of PKC activity vs. calcium concentration. (b) PKC activity vs. PS concentration. (c) Double reciprocal plots of PKC activity vs. ATP concentration.

metals [11] in this region. Parallel experiments designed to examine the interaction with the catalytic site of PKC showed that $[Cu(EpiDXR)_2]$ (Fig. 2(c)) and Cu(II) (data not shown) also exert a non-competitive inhibitory effect on this site.

PKC interaction with Cu(II) and $[Cu(EpiDXR)_2]$ was further investigated by EPR spectroscopy (Fig. 3). No resonance lines were detected at 123 K in ARS (supplemented with buffer A) containing Cu(II) 200 μM (Fig. 3(a)). When PKC was added to the same solution instead of buffer A (final concentration 0.1 μM), the resonance lines of divalent copper in a tetragonal field symmetry became well evident (Fig. 3(c)). 200 μM

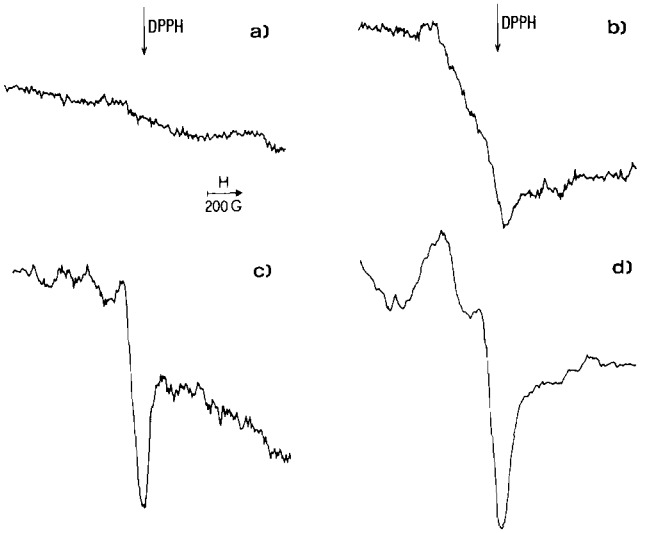

Fig. 3. ESR spectra recorded at 123 K, 5 mW microwave power, 16 G modulation amplitude, 1.25×10^4 receiver gain, on 200 μM solutions of: (a) Cu(II) in ARS; (b) [Cu(EpiDXR)$_2$] in ARS; (c) Cu(II) in ARS containing PKC (0.1 μM); (d) [Cu(EpiDXR)$_2$] in ARS containing PKC (0.1 μM); receiver gain 8×10^3.

[Cu(EpiDXR)$_2$] in ARS showed broad lines due to the isotropic resonances of Cu(II) (Fig. 3(b)), which were widely different from the anisotropic, well resolved lines of the anthracycline complex in aqueous solution [6]; it is conceivable that the stereoelectronic effects responsible for the anisotropic spectrum of [Cu(EpiDXR)$_2$] in water [6–9] are absent in buffer A-containing ARS. The spectrum obtained in the presence of 0.1 μM PKC (Fig. 3(d)) could result from superimposition of the resonances of [Cu(EpiDXR)$_2$] in ARS (Fig. 3(b)) and of divalent copper in PKC-containing ARS (Fig. 3(c)); the overall intensity of the signal is about 50% higher than the sum of the two separate components.

The comparative evaluation of EPR spectra indicates that the interaction of Cu(II) with components of buffer A-supplemented ARS renders the metal ion EPR-silent, possibly due to reduction to Cu(I) by reducing agents present in the system or to formation of spin-coupled Cu(II) derivatives. Whatever the reason for the diamagnetic behavior of copper, the appearance of Cu(II) resonance lines in the presence of PKC suggests that a reaction with the enzyme occurs, which stabilizes an EPR-active paramagnetic enzyme derivative. Coordination with the anthracycline has a similar stabilizing effect on EPR-active Cu(II) centers (Fig. 3(b)). In the presence of PKC, a mutual potentiation of these effects is observed. Since the increase in EPR-active Cu derivatives in the presence of anthracycline and PKC is associated with an enhanced inhibition of the enzyme (as compared to the effects of Cu(II) and EpiDXR alone), we conclude that these paramagnetic Cu(II) centers are involved in the inhibitory effects on PKC.

A comparison of the spectra of [Cu(EpiDXR)$_2$] obtained in the absence (Fig. 3(b)) and presence of PKC (Fig. 3(d)) allows to suggest that PKC is able to remove Cu(II) from its anthracycline complex; Cu(II) shifts from a ligand which stabilizes its paramagnetic form to another with an equivalent action and is thus effectively shielded from possible interaction with ARS components. Formation of a ternary complex Cu(II)–anthracycline–PKC can be ruled out, similarly to what we observed in our studies on the Cu(II)–anthracycline–superoxide dismutase system [18].

In conclusion, we think that the synergistic inhibition of PKC activity observed with Cu and EpiDXR is essentially due to the fact that chelation with anthracycline increases the amount of Cu(II) available for coordination to PKC. According to this hypothesis, the anthracycline–complex can be viewed as a vehicle for Cu(II), from which the metal can be removed by PKC. Since Cu(II) is normally present in *in vivo* systems, its role in PKC modulation and in the inhibitory action of some pharmacological agents should not be overlooked.

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