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LIPID PEROXIDATION, PHOSPHOINOSITIDE TURNOVER AND PROTEIN KINASE C ACTIVATION IN HUMAN PLATELETS TREATED WITH ANTHRACYCLINES AND THEIR COMPLEXES WITH Fe(III)

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Abstract—The effects of the antitumor drugs daunorubicin, doxorubicin and their complexes with Fe(III) on phosphoinositide hydrolysis, lipid peroxidation and protein kinase C (PKC) activation were measured in intact human platelets. Doxorubicin and the Fe(III) complexes of both doxorubicin and daunorubicin quickly induced lipid peroxidation [as measured by the thiobarbituric acid (TBA) assay], phosphorylation of the 40 K substrate of PKC, and increased levels of phosphatidic acid and inositol phosphates. Fe(III) alone or complexed to acetohydroxamic acid induced high levels of TBA-reactive material but did not affect either PKC activation or phosphoinositide turnover. In contrast, daunorubicin, which was ineffective *per se*, inhibited all these doxorubicin- and anthracyclines/Fe(III)-induced biochemical events. We suggest that phosphoinositide hydrolysis determined by anthracyclines, and consequently PKC activation, could be due to lipid peroxidation, thus triggering the activity of phospholipase C.

An increasing importance is being attributed to the interaction of the anthracycline anticancer agents [1, 2] with cell membranes [3]. The effect of such an interaction on the pharmacological activity of these drugs is not understood. A few reports have shown that anthracyclines can be cytotoxic even without entering the cells [4, 5]. Little is known about the functional target(s) of anthracyclines on the plasma membrane. One of these could be the phosphoinositide cycle [6], which is involved in the control of many cellular functions including cell proliferation [7]. Several effectors, among them growth factors, hormones and cytokines, modulate the activity of a phosphoinositide-specific phospholipase C, which produces inositol phosphates and 1,2-diacylglycerol (DAG†) [8]. The former regulate cellular Ca^{2+} homeostasis [9], whereas the latter is the physiological activator of protein kinase C (PKC), a key enzyme in the control of cell differentiation and proliferation [7, 10].

We have shown recently that doxorubicin, but not daunorubicin, is able to activate PKC in intact human platelets [11]. The stimulation was ascribed to the hydrolysis of phosphoinositides because of the inhibitory effect of neomycin [12]. We did not identify the mechanism leading to the breakdown of phosphoinositides by doxorubicin, which is known to bind to negatively charged phospholipids with a high affinity [13]. However, there is much information about the capability of such drugs in modifying the structure of the lipid bilayer by causing peroxidation of unsaturated fatty acids [14, 15]. Since peroxidized lipids appear more susceptible to certain phospholipases [16], lipid peroxidation could be the primary event leading to activation of PKC by the anthracycline. Doxorubicin and daunorubicin differ in their redox properties in that only the former can reduce oxygen directly, whereas daunorubicin requires enzymatic activation [17, 18]. Therefore, the observed differences in PKC activity between the two drugs might well be explained by differences in their ability to peroxidize membrane lipids, as suggested by a parallelism found previously between PKC activation and lipid peroxidation induced in human platelets by doxorubicin analogs [19]. To test this hypothesis, besides the anthracyclines we assayed the activity of their complexes with Fe(III), which are characterized by much higher peroxidizing properties [20] and whose effects on purified platelet membranes have been characterized previously [21].

In this paper we present evidence that in anthracycline-treated platelets the phosphoinositide turnover is enhanced. We suggest that this event could be related to the lipid peroxidizing activity of the drugs.

MATERIALS AND METHODS

Materials. Human blood was provided by the immunohematology department of our Institute. Anthracyclines were a gift from Farmitalia-Carlo Erba (Milan, Italy). Carrier-free [$^{32}P_i$]orthophosphate (10 mCi/mL) and *myo*-[^{3}H]inositol (with PT6-271, 89–120 Ci/mmol) were obtained from Amersham International (Amersham, U.K.). Neo-

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† Abbreviations: DAG, 1,2-diacylglycerol; PKC, protein kinase C; PA, phosphatidic acid; MDA, malondialdehyde; TBA, thiobarbituric acid; PGI_2, prostaglandin I_2; SDS, sodium dodecyl sulfate.

mycin and prostaglandin I_2 (PGI_2) were from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

Drugs were complexed with Fe(III) by rapidly mixing concentrated aqueous solutions containing stoichiometric amounts of reactants. Complexes [2 mol of anthracycline or 3 mol of acetohydroxamic acid per atom of Fe(III)] were diluted to the appropriate concentrations with resuspension buffer (15 mM Tris–HCl, 0.14 M NaCl, 5.5 mM glucose, pH 7.4).

Preparation of platelets. Platelets were isolated from fresh blood of healthy human donors by differential centrifugation. After sedimentation of erythrocytes and nucleated cells (250 *g* for 15 min), platelets were precipitated from platelet-rich plasma (1400 *g* for 5 min). The cells were first washed in acid citrate–dextrose anticoagulant solution, then in washing buffer (4.3 mM K_2HPO_4, 4.3 mM Na_2HPO_4, 24.4 mM NaH_2PO_4, 0.113 M NaCl, 5.5 mM glucose, pH 6.5). PGI_2 (10 ng/mL) was added to platelet suspension before centrifugations [22].

^{32}P-Labeling of platelets. The washed platelets were resuspended in resuspension buffer and incubated for 15 min at 37° as described by Lyons *et al.* [23]. After sedimentation the cells were resuspended in the same medium at a density of 5×10^9 platelets/mL and incubated for 30 min at 37° in the presence of 0.5 mCi/mL of carrier-free [$^{32}P_i$]-orthophosphate and PGI_2.

Protein phosphorylation. $^{32}P_i$-Labeled platelets were treated with drugs or chemicals under the conditions indicated in the figure legends. Each sample contained 5×10^7 cells in 50 μL final volume. All incubations were carried out at 37° and were stopped by addition of 25 μL of concentrated Laemmli sample buffer (0.19 M Tris–HCl, 9% sodium dodecyl sulphate (SDS), 6% 2-mercaptoethanol, 15% glycerol, Bromphenol blue, pH 6.7) [24]. The solubilized platelets were electrophoresed on 12.5% polyacrylamide gels according to the method of Laemmli [24]. An identical amount of platelet extract was applied to each lane of the gels. The gels were fixed for 60 min in 45% methanol and 10% acetic acid in water, for 30 min in 50% methanol and 2% glycerol in water, then dried and autoradiographed with preflashed Cronex-4 Dupont X-ray films. $^{32}P_i$ incorporated into the 40 K bands was evaluated by integration of the photodensitometric patterns of the autoradiograms with an LKB Ultroscan XL laser densitometer.

Phosphatidic acid (PA) determination. Experiments were carried out as described for protein phosphorylation. Reactions were stopped by addition of 0.8 mL of methanol/concentrated HCl (100:6, v/v) and phospholipids extracted with 4 mL of chloroform/methanol/100 mM KCl (2:1:2, by vol.). Separation was performed by TLC on preactivated Silica gel 60 plates (Merck) impregnated with 1.3% potassium oxalate and 2 mM EDTA in methanol/H_2O (2:3, v/v). The plates were developed with chloroform/acetone/methanol/acetic acid/H_2O (40:15:13:12:8, by vol.) [25]. Spots of [$^{32}P_i$]PA were localized by autoradiography, scraped from the plates and counted in a liquid scintillation counter.

Inositol phosphate determination. Washed platelets (2.5×10^9 cells/mL) were labeled in washing buffer for 3 hr with 100 μCi/mL of *myo*-[^{3}H]inositol in the presence of 500 ng/mL PGI_2 and 1 mM EGTA [26]. Cells were then washed with resuspension buffer containing 10 mM LiCl and resuspended in the same buffer. Reactions were performed in 200 μL containing 2×10^8 cells as described in the figure legends. After the appropriate reaction time the samples were treated as described for PA determination and omitting KCl from the extraction solvent. Total inositol phosphates were separated by ion-exchange chromatography on 1-mL columns of AG 1-X8 resin (formate form, Bio-Rad, Richmond, CA, U.S.A.) and eluted with 10 mL of 0.8 M ammonium formate/0.1 M formic acid [26]. An equal volume of Ready Gel (Beckman) was added to each sample for determination of radioactivity.

Lipid peroxidation. The degree of lipid peroxidation was evaluated by measuring the formation of malondialdehyde (MDA) by the thiobarbituric acid (TBA) method [27, 28]. For lipid peroxidation studies the reaction mixtures were the same as for phosphorylation experiments, except that unlabeled platelets were used. The reactions were stopped with 50 μL of TBA reagent (2 vol. 0.8% TBA + 1 vol. 7% perchloric acid in water). After boiling for 10 min, the samples were diluted with an equal volume of 2% SDS and analysed by HPLC in order to separate the MDA adduct, which was read at 532 nm, from interfering anthracyclines. One hundred microlitres was injected into a 4.6 × 300 mm C18 μBondapack column. Elution was performed with 0.5% SDS in 10% acetonitrile at a flow rate of 1.5 mL/min. Elution was monitored at 532 nm with a Perkin–Elmer LC-95 spectrophotometer detector and the signal was integrated with a Waters 740 data module.

RESULTS

Treatment of intact platelets with doxorubicin resulted in phosphorylation of the 40 K substrate of PKC [11, 29] associated with a dose-dependent production of MDA, as shown in Fig. 1A. This behaviour was shared, among the tested anthracyclines, also by 4′-iodo-4′-deoxydoxorubicin [19]. Daunorubicin did not cause any significant increment above the basal values of 40 K phosphorylation and TBA-reactive material. Then we assayed the effects of the 2:1 (drug/Fe) preformed complexes of doxorubicin and daunorubicin to verify whether the correlation between 40 K phosphorylation and lipid peroxidation was maintained. Figure 1B shows that, when complexed with iron, daunorubicin acquired the ability to induce PKC activation and lipid peroxidation. Moreover, the effects of both drugs complexed with Fe(III) were very similar and amplified with respect to doxorubicin alone.

Fe(III) induced high levels of lipid peroxidation, alone and complexed with acetohydroxamic acid. However, neither Fe(III) nor the complex, which is able to enter the cells [30], induced significant amounts of 40 K phosphorylation under our experimental conditions (Fig. 1C).

Daunorubicin inhibited doxorubicin-induced 40 K phosphorylation in human platelets, as shown

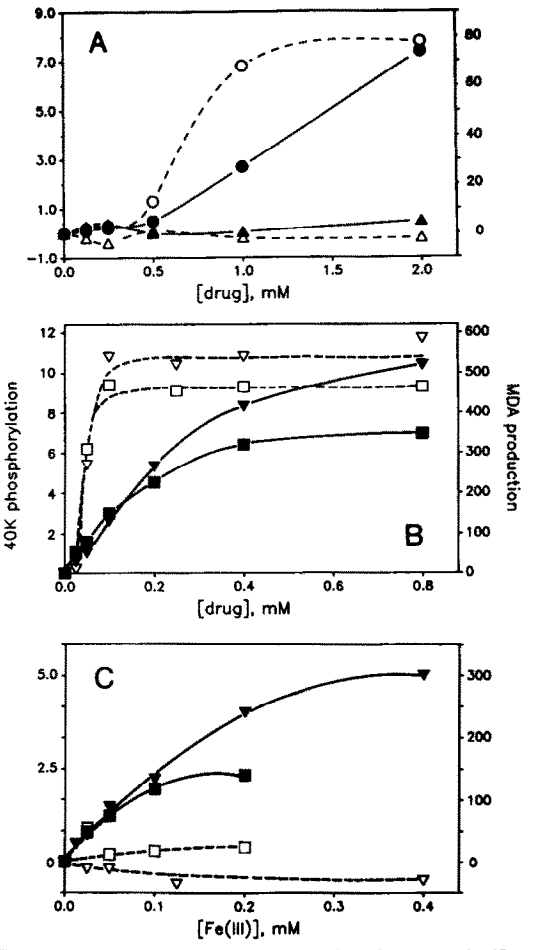

Fig. 1. Dose–response of 40 K phosphorylation and MDA production induced by several effectors. Washed platelets ($^{32}P_i$-labeled for phosphoprotein analysis) were incubated for 3 min with the indicated concentrations of drug, then 40 K phosphorylation (dashed lines) and MDA production (solid lines) were determined as described in Materials and Methods. Results are expressed as percentage of total [^{32}P]-phosphoproteins and as picomoles of MDA/10^8 platelets, respectively. Control values were subtracted from each point. Data shown in this and all following figures are from a representative experiment. MDA determinations were performed in duplicate or triplicate. Panel A: (●, ○) doxorubicin; (▲, △) daunorubicin. Panel B: (▼, ▽) doxorubicin/Fe(III); (■, □) daunorubicin/Fe(III). Panel C: (▼, ▽) Fe(III); (■, □) acetohydroxamic acid/Fe(III).

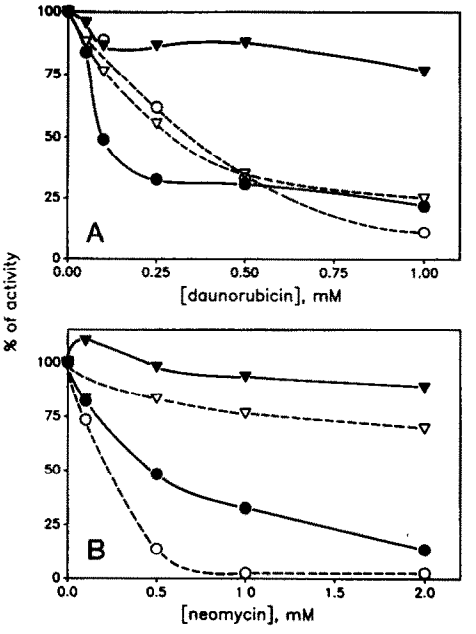

Fig. 2. Inhibition by daunorubicin (A) and neomycin (B) of 40 K phosphorylation (dashed lines) and MDA production (solid lines) induced by several effectors. Platelets were preincubated for 5 min with the indicated concentrations of inhibitors, then treated for 3 min with 1 mM doxorubicin (●, ○) and 0.2 mM doxorubicin complexed to 0.1 mM Fe(III) (▼, ▽). Results are expressed as percentage of the effect observed in the absence of the inhibitor. Values of control samples treated with the same inhibitor concentrations were subtracted. Superimposable curves were obtained with daunorubicin/Fe(III) as activator.

previously [11]. The drug also inhibited (Fig. 2A) the MDA production stimulated by doxorubicin, as well as the PKC activation induced by the complexes. Although the percentage inhibition of complex-induced MDA production was less appreciable, its absolute reduction (70 pmol at 1 mM daunorubicin) was higher than in free drug-activated platelets (25 pmol at the same daunorubicin concentration). Daunorubicin did not prevent lipid peroxidation by Fe(III) (data not shown). The possibility that a peroxidating drug/Fe(III) complex could have been formed during incubation of daunorubicin-pretreated platelets with Fe(III) was ruled out by the lack of PKC activation under the same experimental conditions (not shown).

The possibility of a PKC-induced lipid peroxidation [31] was evaluated by treating platelets with 12-*O*-tetradecanoylphorbol-13-acetate, a direct PKC activator [32]. Doses of the tumor promoter (80 and 200 nM) that give levels of 40 K phosphorylation higher than 1 mM doxorubicin did not raise the MDA levels above the control values (Fig. 3). Platelet activation by a physiological stimulus such as thrombin is always associated with MDA production, a by-product of thromboxane formation through the phospholipase A_2/cyclooxygenase pathway. In order to assess a direct effect of doxorubicin on lipid peroxidation, platelets were treated with 20 μM indomethacin and stimulated with thrombin, doxorubicin and its complex with Fe(III). Figure 3 shows that, in contrast to thrombin, MDA production induced by doxorubicin/Fe(III) complex and by 0.5 mM doxorubicin was apparently unaffected, while at the highest doxorubicin concentration only a partial inhibition was observed under our experimental conditions. Indomethacin did not affect 40 K phosphorylation induced by the same activators (not shown). Since it has been shown that doxorubicin

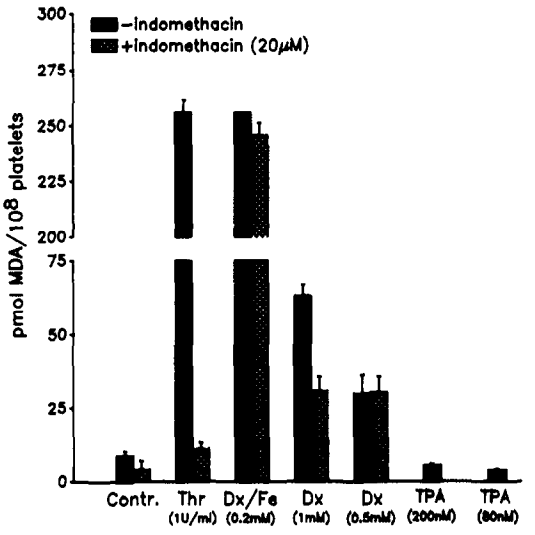

Fig. 3. Lipid peroxidation in indomethacin-treated platelets. Platelets were incubated with 20 μM indomethacin before treatment for 3 min with the indicated effectors [Thr, thrombin; Dx, doxorubicin; Dx/Fe, 2:1 complex of doxorubicin with Fe(III)].

and the complex of this drug with Fe(III) are inhibitors of PKC activity in cell-free systems [33, 34], the activation of the enzyme that we observed in intact platelets could be the consequence of phospholipase action, whose activity is known to be favoured by lipid peroxidation [15, 16].

A previous study, in which neomycin was used as an inhibitor of phospholipase C [12], suggested that doxorubicin-induced PKC activation in platelets could be due to phosphoinositide hydrolysis and the consequent production of DAG [11]. As shown in Fig. 2B, inhibition by the drug of doxorubicin-induced 40 K phosphorylation was also associated to a marked reduction in MDA production. In contrast, doxorubicin/Fe(III)-induced effects were only slightly affected by neomycin. It was therefore necessary to verify the hypothesis of an anthracycline-driven phosphoinositide hydrolysis, at least to explain the effects of the complexes.

Activation of PKC still appeared to be due to DAG, since synthesis of PA, i.e. DAG [26], was also induced when PKC activation occurred. Figure 4 shows the time-course of PA production in $^{32}P_i$-labeled platelets stimulated with doxorubicin and the Fe(III) complexes of doxorubicin and daunorubicin. The dose dependency of the effect, which is consistent with the observed 40 K phosphorylation (Fig. 1A and B), is shown in Fig. 5. Since the hydrolysis of other lipids can generate DAG [35] but only phosphoinositides can produce inositol phosphate, this parameter was also evaluated. The production of inositol phosphates in platelets treated with free and complexed drugs is reported in Fig. 6 The data confirm the complete inactivity of daunorubicin and show that inositol phosphate production almost parallels PA synthesis. This demonstrates the involvement of phosphoinositide

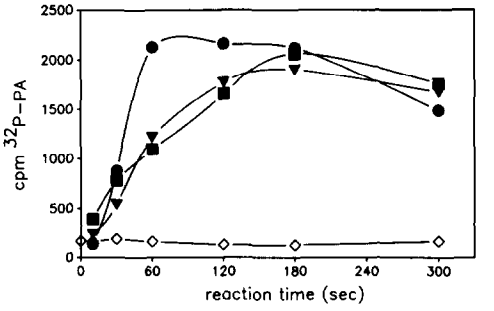

Fig. 4. Time-course of PA induction by anthracyclines. Platelets were treated with 1 mM doxorubicin (●), 0.2 mM doxorubicin/Fe(III) (▼) and 0.2 mM daunorubicin/Fe(III) (■) for the indicated times. PA was extracted and evaluated as described in Materials and Methods. Untreated control platelets were processed similarly (◇).

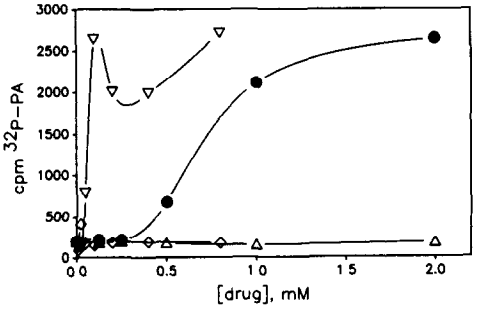

Fig. 5. Dose–response curves of PA induction by anthracyclines. Platelets were treated for 3 min with the indicated concentrations of doxorubicin (●), daunorubicin (△), doxorubicin/Fe(III) (▽) and Fe(III) (◇). PA was determined in duplicate as described in Materials and Methods.

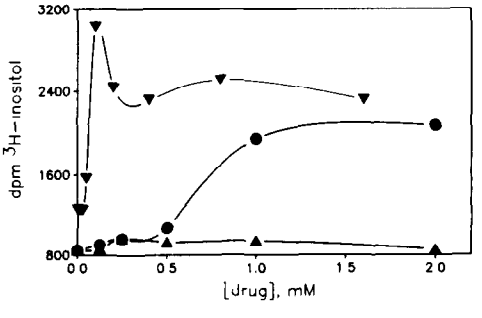

Fig. 6. Dose–response of inositol phosphate production by anthracyclines. [^{3}H]Inositol prelabeled platelets were treated for 3 min with the indicated concentrations of doxorubicin (●), daunorubicin (▲) and doxorubicin/Fe(III) (▼). Total inositol phosphates were extracted and counted as described in Materials and Methods. Data shown are the average of triplicate values.

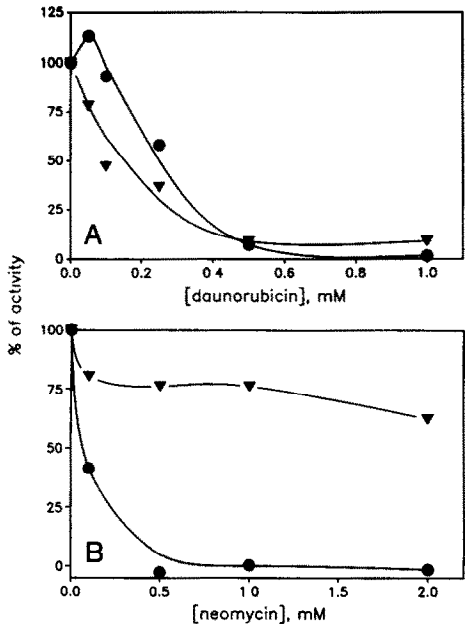

Fig. 7. Inhibition by daunorubicin (A) and neomycin (B) of PA synthesis induced by 1 mM doxorubicin (●) and 0.2 mM doxorubicin complexed with Fe(III) (▼). Platelets were treated as described in the legend to Fig. 2, then PA was determined as described in Materials and Methods. Results are expressed as per cent of the effect observed in the absence of the inhibitor.

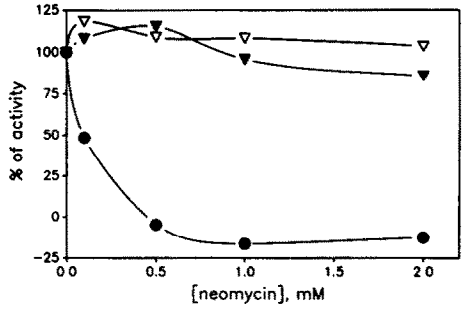

Fig. 8. Effect of neomycin on inositol phosphate production induced by 1 mM doxorubicin (●) and by 0.8 mM (▼) or 0.2 mM (▽) doxorubicin complexed with Fe(III). Platelets were treated as described in the legend to Fig. 2 and total inositol phosphates determined as described in Materials and Methods. The average of duplicate values is expressed as a percentage of the effect observed in the absence of the inhibitor.

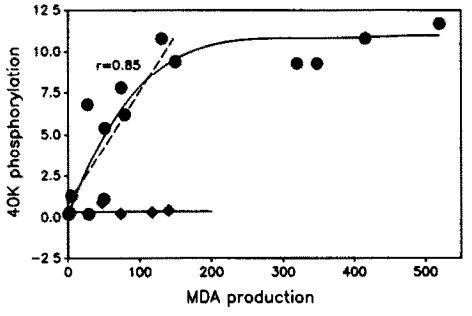

Fig. 9. Relationship between MDA production and 40 K phosphorylation. 40 K phosphorylation induced by doxorubicin and Fe(III) complexes of doxorubicin and daunorubicin (●) is plotted versus the corresponding MDA production (data from Fig. 1). For data up to 120 pmol MDA, the linear regression was calculated (dashed line). The plot of Fe(III)/acetohydroxamic acid is also reported (◆).

breakdown in PKC activation induced by doxorubicin but also by drug/Fe(III) complex, despite the lack of an inhibitory effect of neomycin on 40 K phosphorylation induced by the complex itself. The effect of daunorubicin on PA synthesis was also determined. As found for MDA production and 40 K phosphorylation (Fig. 2A), PA synthesis induced by doxorubicin and doxorubicin/Fe(III) was inhibited by daunorubicin (Fig. 7A). Neomycin once again failed to reduce PA synthesis (Fig. 7B) and inositol phosphate production induced by the complex (Fig. 8), whereas the predicted effect was maintained when the activator was free doxorubicin.

DISCUSSION

The results presented here demonstrate that the ability of doxorubicin and anthracycline/iron complexes to activate PKC in intact human platelets [11] is due to stimulation of phosphoinositide hydrolysis, possibly caused by drug-induced lipid peroxidation. The relationship between MDA production and 40 K phosphorylation is illustrated in Fig. 9. In the graph the data from Fig. 1 are summarized by reporting 40 K phosphorylation as a function of MDA synthesis irrespective of the activator. There appears to be a linear relationship up to about 120 pmol of MDA, after which the stimulus reaches saturation and no further increase in 40 K phosphorylation is observed. Complexes of Fe(III) with acetohydroxamic acid, which unlike Fe(III) alone can enter the cells [30], were able to peroxidize lipids but not to activate PKC, thus indicating that the proposed relationship is only valid for the tested anthracyclines and their Fe(III) complexes. Since the binding of doxorubicin to phospholipids has been demonstrated [13, 36], it is therefore conceivable that peroxidation of the unsaturated acyl chains of these molecules causes the activation of phospholipases [15, 16] and the production of DAG, which in turn brings about the activation of the enzyme.

Such a model is supported by our data showing that all activators inducing lipid peroxidation and 40 K phosphorylation were able to cause synthesis of PA. If we assume that DAG kinase is not highly affected by the drugs, then the PA formed is representative of the DAG generated [26] and its relative amount is congruent with the observed

amount of 40 K phosphorylation. Phosphoinositides are not the only source of DAG [35]. Although we cannot exclude the hydrolysis of other phospholipids [37], the remarkable production of inositol phosphates clearly indicates that a phosphoinositide-specific phospholipase C was activated in platelets treated with doxorubicin or anthracycline/Fe(III) complexes. The PA and inositol phosphate curves produced by the complexes are not simple and show a maximum at 100 μM of drug (Figs 5 and 6). This is not an artifact because the same plot was reproduced several times. An explanation for the reduced effect of the complexes at concentrations above 100 μM might be a massive impairment of membrane structure that restrains the activity of phospholipase C.

Although our data strongly support the proposed model, they do not provide a conclusive demonstration of a causal relationship between lipid peroxidation and PKC activation. This is mainly because, especially in the presence of iron ions, the TBA-assay might be affected by a high background due to peroxide decomposition during the heating step [38]. This might explain the high peroxidizing rates of Fig. 3 and also the fact that under our experimental conditions we do not find any difference between the peroxidizing ability of doxorubicin/ and daunorubicin/iron complexes, while on isolated membranes the latter has been reported to be much less active [21]. On the other hand, the response of PKC is the same to both complexes also at non-saturating doses, thus suggesting different substrates for peroxidation in intact platelets. Our attempts to separate peroxidation from phosphorylation by using neomycin were unsuccessful because the aminoglycoside, which binds to phosphoinositides with a high affinity and inhibits the activity of phospholipase C [12], also reduced lipid peroxidation induced by doxorubicin. Moreover, although the production of PA and inositol phosphates clearly indicated phosphoinositide hydrolysis, neomycin only slightly inhibited complex-induced effects. Similar results were obtained with indomethacin that did not inhibit doxorubicin-induced 40 K phosphorylation (not shown) and reduced only partially the MDA production. Although the last effect could be artifactual, taken together these data exclude a pathway of PKC activation by anthracyclines and their Fe(III) complexes through arachidonic acid metabolism [39].

Certainly, MDA production is not secondary to activation of the enzyme [31], since in our experimental conditions 12-*O*-tetradecanoylphorbol-13-acetate concentrations as high as 200 nM, giving massive 40 K phosphorylation, did not raise MDA significantly above control levels.

In conclusion, the data presented here confirm that activation of PKC in anthracycline-treated platelets is a consequence of increased phospholipid turnover. This effect, that was also obtained in Swiss 3T3 fibroblasts [40], appears to be directly proportional to the ability of the inducer to cause lipid peroxidation, measured with the TBA assay. The concentrations necessary to observe such effects in platelets and in nucleated cells are far higher than cytotoxic ones. However, since anthracycline-induced lipid peroxidation has been reported *in vivo* at therapeutic doses [41, 42], we are investigating the possibility that such events have a role in modulating the pharmacological properties of these drugs. For this study the coarse TBA assay is clearly inadequate and more sensitive and specific assays for determining the particular lipids involved in the peroxidative process need to be applied.

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