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DIVERSITY OF EFFECTS OF TWO ANTITUMOR ANTHRACYCLINE ANALOGS ON THE PATHWAY OF ACTIVATION OF PKC IN INTACT HUMAN PLATELETS*

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Abstract—Two antitumor antibiotics doxorubicin and daunorubicin were tested for their ability to influence the activation of protein kinase C in human platelets. Daunorubicin was found to inhibit the phosphorylation of the 40 K PKC substrate induced by thrombin and 12-*O*-tetradecanoyl-phorbol-13-acetate as well as the phosphorylation of the 20 K protein induced by thrombin. The serotonin release associated to these phosphorylative events was also inhibited by daunorubicin. In contrast the effects of doxorubicin, though inhibitory on the release reaction, were always stimulatory of the phosphorylations. Doxorubicin alone was able to induce the phosphorylation of both 40 K and 20 K phosphoproteins in a concentration-dependent manner. Whereas the stimulation by doxorubicin was not influenced by pretreatment with dibutyril-cyclic-AMP which inhibits the effects of thrombin, this effect was inhibited by daunorubicin, neomycin and stimulated by the diacylglycerol-kinase inhibitor R 59 022. It is proposed that doxorubicin activates the protein kinase C by causing the breakdown of phosphoinositides.

Doxorubicin‖ (formerly known as adriamycin) and daunorubicin are among the most powerful chemotherapeutic agents presently available for cancer therapy. The two structurally similar drugs [1] differ in the cytotoxic activity [2, 3] and in therapeutic effectiveness [4]. These differences in their biological properties have not received a definitive explanation at the molecular level. The mechanism of action of these and other active members of the anthracycline family is still a matter of discussion because of the multiplicity of targets that have been proposed to explain their cytotoxic and antitumor activities.

Although DNA remains the main target for these intercalating agents, it has been shown that DX can bind, even covalently, to proteins [5–7]. Furthermore many reports indicate in cellular membranes a possible additional target of anthracycline action (reviewed in Ref. 8). Alteration of membrane fluidity [9], lipid peroxidation [10], *in vitro* binding to phospholipids [11, 12] and interference on membrane associated enzymatic activity [13, 14] have been described. Recently a cytotoxic effect of DX has been shown in systems where the drug could only interact with the cell membrane [15–17]. This membrane-mediated cytotoxic activity is a quite interesting phenomenon since many biochemical pathways responsible for transducing extracellular stimuli reside in the plasma membrane. One of these is the phosphoinositide cycle [18] whose stimulation leads to the production of DG and to the activation of PKC, a key enzyme in the mechanisms regulating cell proliferation [19] and differentiation [20]. Since the biochemical activities of some oncogene products appear to be correlated with the phosphoinositide pathway [21–24] and PKC activation [25, 26], the aim of this work was to investigate about a possible interference of these antitumor antibiotics on the membrane mechanisms leading to the activation of PKC. Platelets were chosen as a model for in this system the activation of PKC is easily identified by the phosphorylation of the 40 K protein [27]. The main result of this work has been the finding that DX can induce specific protein phosphorylation in platelets while the effects of DN are inhibitory of the phosphorylations induced by thrombin.

MATERIALS AND METHODS

Materials. Human blood was provided by the Immunohematology Division. DX and DN were a gift from Farmitalia-Carlo Erba (Milan, Italy). Bovine thrombin was purchased from Boehringwerke, TPA, DBcAMP and neomycin from SIGMA, and the diacylglycerol kinase inhibitor R 59 022 from Jannsen. *Staphylococcus aureus* V8 protease and bovine α-chymotrypsin were from Miles Laboratories while bovine trypsin was a product from Worthington Diagnostics. [^{14}C]Serotonin and carrier-free [^{32}P]orthophosphate were obtained from Amersham International (U.K.).

Preparation of platelets. Platelets were isolated

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‖ Abbreviations used: DX, doxorubicin; DN, daunorubicin; PKC, Ca^{2+}-activated phospholipid-dependent protein kinase; CMK, Ca^{2+}/calmodulin-dependent protein kinase; DG, diacylglycerol; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; IP_3, inositol trisphosphate; DBcAMP, N^6,2′-*O*-dibutyryladenosine-3′:5′-cyclic monophosphate; PLC, phospholipase C; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

from fresh blood of healthy human donors by a modification of the method of Baezinger and Majerus [28]. After the separation by differential centrifugation, 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).

^{32}P labeling of platelets. The washed platelets were resuspended in a phosphate-free resuspension buffer (15 mM Tris–HCl, 0.14 M NaCl, 5.5 mM glucose pH 7.4) and incubated for 15 min at 37° as described by Lyons *et al.* [29]. 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]orthophosphate.

Protein phosphorylation. ^{32}P-labeled platelets were treated with drugs or chemicals under the conditions indicated in the figure legend. Each sample usually contained 3.75×10^8 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% SDS, 6% 2-mercaptoethanol, 15% glycerol, bromphenol blue, pH 6.7).

SDS-PAGE. The solubilized platelets were electrophoresed on 15% polyacrylamide gels according to the method of Laemmli [30] using a 6% stacking gel. An amount of platelet extract derived from an identical number of cells was applied to each lane of the gels. The gels were fixed for 60 min in 45% methanol, 10% acetic acid in water, for 30 min in 50% methanol/2% glycerol in water then dried and autoradiographed with a 3M Trimax X-ray film. ^{32}P incorporated into the 40 K and 20 K bands was evaluated by integration of the photodensitometric pattern of the autoradiograms with an LKB Ultroscan XL laser densitometer.

Peptide mapping. The partial proteolytic digestion of the phosphorylated 40 K proteins was performed as described by Cleveland *et al.* [31] using the procedure for proteins in gel slices. The 40 K bands were localized in the gel from the autoradiograph, cut out and applied to a second 15% gel in the presence of 10 μg of trypsin, α-chymotrypsin or *Staphylococcus aureus* V8 protease. The digestion was allowed for 60 min interrupting the run as the dye reached the separating gel then the run was resumed. The gel was then fixed, dryed and autoradiographed.

Serotonin release. A suspension of 6×10^8 platelets/ml in washing buffer was incubated with [^{14}C]-serotonin (0.15 μCi/ml) at 37° for 60 min. The platelets were then sedimented and resuspended in the same buffer at a density of 4×10^9 cells/ml. The release reaction was performed diluting the platelets 1 : 10 in resuspension buffer containing various concentrations of drugs, preincubating for 5 min at 37° then adding 0.1 U/ml of thrombin dissolved in a small volume of resuspension buffer. At the end of incubation the reaction was stopped by adding formaldehyde to 1.5% final concentration as reported by Costa and Murphy [32]. Samples were then centrifuged at 12,000 *g* for 40 sec and released radioactivity determined in the supernatant by liquid scintillation counting.

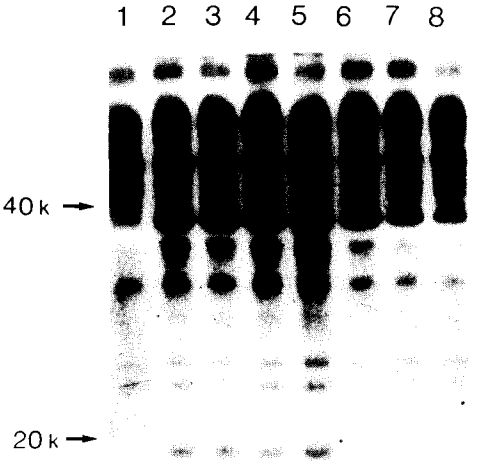

Fig. 1. Effect of DX and DN on thrombin-induced protein phosphorylation. ^{32}P pre-labeled platelets were incubated in the presence of various concentrations of the anthracyclines at 37° for 5 min and then stimulated with 0.1 U/ml thrombin for 90 sec. The details of the experiment are described in Materials and Methods. Samples were separated by SDS-PAGE and ^{32}P-labeled proteins visualized by autoradiography. The positions of the 40 K and 20 K phosphoproteins were determined relatively to ^{14}C-labeled standard proteins (not reported). Lane 1: control; lane 2: thrombin only; lanes 3–5: thrombin after preincubation of platelets with 0.1, 0.5, and 1 mM DX respectively; lanes 6–8: thrombin after preincubation of platelets with 0.1, 0.5 and 1 mM DN.

RESULTS

Activation of platelets by thrombin is accompanied by polyphosphoinositide breakdown whose products DG and IP_3 are thought to mediate the PKC and CMK activation [33]. While DG acts as the physiologic activator of PKC, IP_3 is responsible for the calcium liberation from internal stores allowing the increase in calcium ions necessary for the CMK activity. Activation of these two kinases can be recognized by the phosphorylation of their specific substrates, the 40 K protein for PKC [27] and the 20 K protein for the CMK [34, 35]. The former has recently been identified as the inositol trisphosphate 5′-phosphomonoesterase [36] while the latter is known as the myosin light chain [35]. Platelets were exposed to the anthracyclines in the presence of thrombin after labeling with ^{32}P. Phosphoproteins were analyzed by SDS-PAGE and revealed by autoradiography of the dried gel. Figure 1 shows that thrombin-induced phosphorylation of the 40 K and 20 K proteins is slightly increased by DX while DN reduces both in a dose-dependent manner. In experiments performed in the absence of activators an opposite effect was still observed after exposure to the two drugs. Figure 2 shows that while DX itself can stimulate the phosphorylation of both 40 K and 20 K proteins, DN alone had no effect. These autoradiograms, overexposed in order to reveal even minor components, show that two peptides of molecular weight lower than 40 K are modulated by

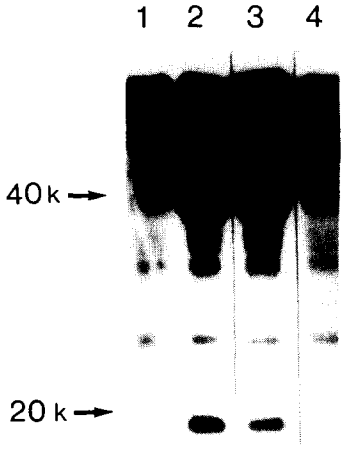

Fig. 2. Effect of DX and DN on protein phosphorylation in intact platelets. Platelets were processed as described in Materials and Methods and Fig. 1. The autoradiogram shows the tracings of ^{32}P-labeled phosphopeptides in platelets exposed to buffer (lane 1), 2 U/ml thrombin for 1 min (lane 2), 1 mM DX for 10 min (lane 3) and 1 mM DN for 10 min (lane 4).

thrombin and DX. Their amount is very little though, and their phosphorylation always parallels that of 40 K. We believe these peptides to be proteolytic products of 40 K and they will not be considered any further.

The 40 K proteins phosphorylated after thrombin or DX were analyzed through the peptide mapping by partial proteolytic digestion (Fig. 3). Although the protein in lane 1 was not sufficiently digested by trypsin the patterns obtained with the other two proteases were superimposable thus supporting the identity of the two proteins. The extent of DX-induced 40 K phosphorylation was dose dependent as evaluated by integration of the autoradiographic tracings (Fig. 4A). At the lower dose of DX tested

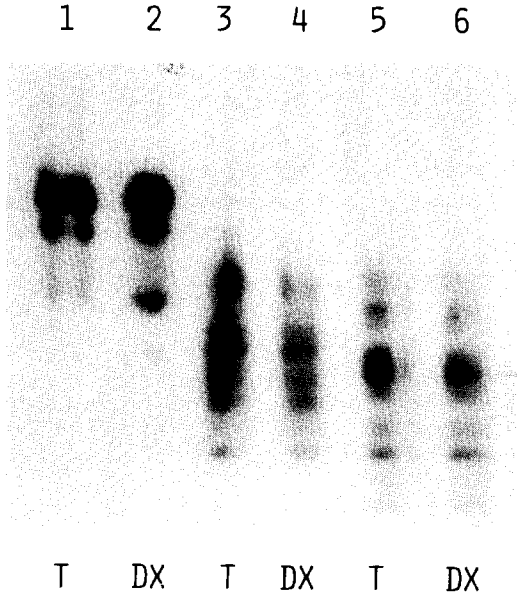

Fig. 3. Comparison of the proteolytic products of 40 K proteins phosphorylated following exposition to thrombin (T) or DX. Slices containing the 40 K bands were excised from a gel similar to that shown in Fig. 2 and subjected to SDS-PAGE in the presence of 10 μg of trypsin (lanes 1, 2), α-chymotrypsin (lanes 3, 4) or staphylococcal V8 protease (lanes 5, 6).

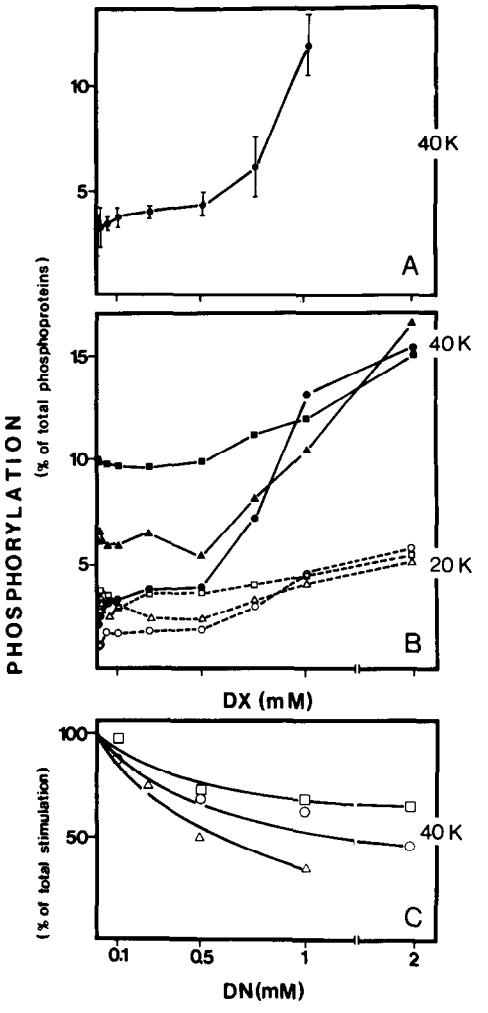

Fig. 4. (A) Dose response of 40 K phosphorylation induced by DX. ^{32}P-labeled platelets were stimulated with different concentrations of drug at 37° for 2 min. ^{32}P containing peptides were separated by SDS-PAGE and revealed by autoradiography. The effect of DX is reported as the per cent of phosphorylated 40 K with respect to total phosphoproteins. Each point is the average (±SD) of two different experiments. (B) Effect of DX on thrombin induced protein phosphorylation. The experiment was carried out as in (A) but incubations were in the presence of DX alone (●, ○) or DX plus 0.1 U/ml (▲, △) or 0.2 U/ml (■, □) thrombin given simultaneously. (C) Reduction of 40 K phosphorylation induced by activators in the presence of DN. ^{32}P-prelabeled platelets were incubated with DN at 37° for 5 min and then stimulated with 1 mM DX (△), 0.2 U/ml thrombin (○) or 100 nM TPA (□) for 90 sec. Data are reported as the percent of 40 K phosphorylation induced by the activator in the absence of DN. Curves in (B) and (C) are representative of different experiments giving similar results.

(10 μM) the 40 K phosphorylation was 10% above the value of untreated platelets. This increase was statistically significant ($P < 0.05$) as calculated by the Student's *t*-test for paired samples. The effect of thrombin was enhanced by DX but this increase was not additive suggesting that thrombin and DX were acting on different steps of the same pathway. It is evident from Fig. 4B that in the presence of both activators the maximum level of phosphorylation of both 40 K and 20 K proteins was comparable to that induced by the anthracycline alone. This would suggest that as DX concentration increases the 40 K phosphorylation is enhanced but the activity of thrombin is concomitantly quenched. Since DX mimicked thrombin, the capacity of DN of inhibiting the anthracycline-induced 40 K phosphorylation was examined. As shown in Fig. 4C DN could reduce the effect of DX as well as that of thrombin and TPA, a tumor promoter of the phorbol ester family which directly activates PKC presumably by substituting for DG [37]. It is worth noting that phosphorylation of the 20 K too is inhibited by DN (data not shown).

It has been described [38] that receptor-mediated PKC activation by thrombin is inhibited by cyclic nucleotides while the direct activation mechanism of diacylglycerol or TPA is not. To obtain clues about the steps affected by DX the effects of thrombin, TPA and DX in the presence of DBcAMP were compared. Results from these experiments (Fig. 5) show that although DX, like thrombin, could induce the phosphorylation of both 40 K and 20 K, these effects were not sensitive to the cyclic nucleotide inhibition resembling in this aspect the mode of PKC activation by TPA. However, the finding that DX could also stimulate the phosphorylation of the myosin light chain suggested the possibility that, differently from TPA, DX did not affect PKC directly since the activity of PKC and CMK is dependent on phosphoinositide metabolism in platelet activation by thrombin. We assayed the eventuality that the drug could enhance the phosphoinoside breakdown by using a diacylglycerol kinase inhibitor and the antibiotic neomycin. The first product, R 59 022, previously described by others [39], amplifies the effects of endogenous DG in platelets by inhibiting its recycling to phosphatidic acid. Neomycin binds to phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate and it was reported to block the phosphoinositide metabolism [40]. In our experiments DX induced 40 K phosphorylation was effectively and reproducibly increased in the presence of R 59 022 in a dose-dependent fashion (Fig. 6). On the contrary neomycin had an inhibitory effect (Fig. 7) thus supporting the hypothesis that the anthracycline can induce PKC activation by causing DG production and intervening on the phosphoinositide pathway.

Phosphorylation of the 40 K and 20 K proteins is a phenomenon constantly associated with the secretory processes in platelets [41]. In consideration of the above observations DX too was expected to produce such a cellular response. The data reported

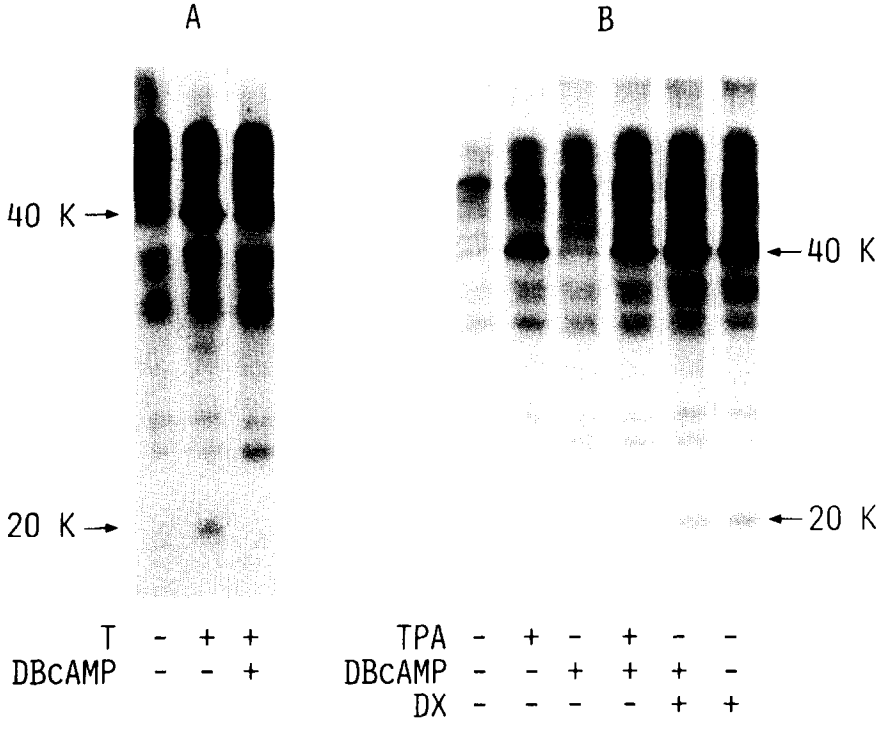

Fig. 5. Effect of DBcAMP on protein phosphorylation induced by thrombin, TPA and DX. ^{32}P-labeled platelets were incubated at 37° in the presence of buffer (−) or 0.5 mM DBcAMP (+) then stimulated as indicated. (A) 0.1 U/ml thrombin or buffer for 90 sec; (B) 150 nM TPA or 1 mM DX for 90 sec; control platelets received the same volume of TPA solvent. All samples were then processed as described in Materials and Methods and Fig. 1.

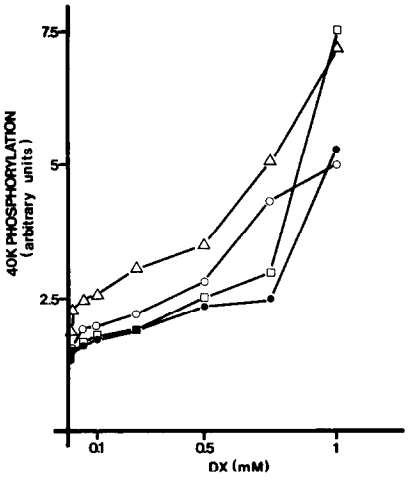

Fig. 6. Stimulation of DX-induced 40 K phosphorylation by R 59022. ^{32}P-labeled platelets were added to RB containing DX alone (●), at the concentrations indicated in abscissa, or DX plus 20 μM (□), 50 μM (○) or 100 μM (△) R 59 022. After two min at 37° the reaction was stopped and the samples processed as described in Materials and Methods and Fig. 1. Curves are representative of experiments giving similar results.

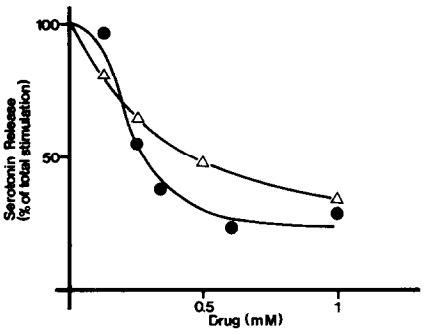

Fig. 8. Inhibition of thrombin induced serotonin release by anthracyclines. Platelets were loaded with [^{14}C]serotonin as described. After a preincubation in the presence of DX (●) or DN (△), at the concentrations indicated in abscissa, at 37° for 5 min, 0.1 U/ml of thrombin was added. After 2 min the reaction was stopped by addition of 16.5% formaldehyde followed by centrifugation at 12,000 *g*. Released serotonin was determined in the supernatant by liquid scintillation counting. Radioactivity released from control platelets was subtracted. The percentage is referred to the release induced by 0.1 U/ml thrombin in the absence of drugs. The arrow indicates the entity of [^{14}C]-serotonin released by stimulation with 1 mM DX alone for 7 min.

in Fig. 8 illustrate the effect of DX and DN on thrombin-induced serotonin release in [^{14}C]serotonin preloaded platelets. It appears that the effect of thrombin is inhibited by both the anthracyclines as already reported in the literature [42, 43]. DX is able to elicit a release reaction; however, the effect is very weak and it needs concentrations higher than 0.5 mM, reaching a plateau after 5 min of incubation (data not shown). In accordance with the effect on protein phosphorylations DN did not cause any serotonin release (data not shown).

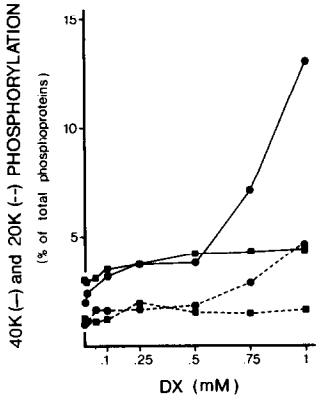

Fig. 7. Inhibition of DX-induced protein phosphorylation by neomycin. ^{32}P-labeled platelets were exposed to DX, alone (●) or in the presence of 0.25 mM neomycin (■) at 37° for 2 min. Labeled phosphoproteins were analyzed by SDS-PAGE followed by autoradiography of the dried gel. The intensity of 40 K and 20 K bands was evaluated by integration of densitometric tracings.

DISCUSSION

Our results show that membrane-associated biochemical pathways involved in the agonist-induced generation of second messengers are affected by both DX and DN. Despite the chemical similarity, the effects of the two drugs on protein phosphorylation in platelets were opposite.

An inhibitory activity would not have been surprising since both drugs have been shown to interact with proteins [5–7] and phospholipids *in vitro* [11, 12] and with synthetic and natural membranes [44, 45]. Furthermore it was reported that PKC activation was inhibited by DX in cell-free systems [46, 47] while Thompson *et al.* [48] recently suggested that DX inhibits the metabolism of a pool of phosphoinositides responsible for the maintenance of the erythrocyte shape. In platelets we found indeed an inhibitory effect of both drugs on thrombin-induced serotonin release, a phenomenon which requires PKC activation and Ca^{2+} mobilization [49]. DN also inhibited protein phosphorylation induced by effectors such as thrombin and TPA. Surprisingly DX not only did not inhibit the effect of thrombin but elicited, alone, a thrombin-like effect on platelets causing the phosphorylation of both 40 K and 20 K proteins. This is to our knowledge one of the most relevant qualitative differences in the biochemical behaviour of these two antibiotics so far described. The details of the mechanisms activated by the interaction of thrombin with its receptor and ending up with the phosphorylation of the 40 K and 20 K proteins are not clearly defined. It is likely that the action of thrombin induces the activation of PLC through a GTP binding protein [50]. PLC generates DG and variously phosphorylated inositol phos-

phates the best known of which, IP_3, has the function of mobilizing calcium ions from internal stores. The increase in calcium concentration allows the activation of CMK which phosphorylates the 20 K protein. The DG in turn activates the PKC thus causing the phosphorylation of its 40 K substrate. Virtually any step of this complex pathway can be affected by these membrane interacting drugs and it is likely that both of them affect more than one. DX causes the phosphorylation of both 40 K and 20 K proteins. This could be explained by different mechanisms:

(1) *Interference with thrombin receptors thus producing a thrombin like effect.* This hypothesis can be excluded by the fact that DBcAMP, while inhibiting completely the effect of thrombin, is without effect on DX-induced protein phosphorylation.

(2) *Direct PKC activation similarly to TPA.* This mechanism would be denied by the finding that DX causes the phosphorylation of both 40 K and 20 K proteins. If this cannot be considered a conclusive argument since it has been shown that the myosin light chain can be directly phosphorylated by PKC [51], we believe this possibility unlikely because in our experimental conditions only DX induces a substantial 20 K phosphorylation when similar amounts of 40 K protein are phosphorylated following DX or TPA treatment (Fig. 5). More convincing in excluding a direct activation of PKC is the reported inhibitory activity of DX on purified PKC *in vitro* [46, 47]. This inhibition has been ascribed to the binding of the drug to phosphatidylserine, an essential cofactor for the enzyme.

(3) *Hydrolysis of phosphoinositides by activation of PLC.* This hypothesis is supported by the amplification of the effects of DX by the diacylglycerol kinase inhibitor R 59 022 and by the effect of neomycin that, inhibiting the activity of PLC by binding to its specific substrates, greatly reduces the effect of DX on protein phosphorylation. Activation of PLC could be attributed to the modification of the phospholipid environment of this enzyme and/or to a specific interaction with the phosphoinositide substrates. The latter are expected to be targets for the anthracyclines as known for other negatively charged phospholipids [11]. However the action of DX on these membrane-associated events seems to be quite complex as indicated by the lack of additivity with thrombin on protein phosphorylation at non saturating concentrations and the concomitant inhibition of serotonin release. In addition, the possibility cannot be excluded on the bases of our data that the observed effects of DX might also be due in part to hydrolysis of phospholipids other than phosphoinositides or to a direct calcium mobilization.

The interference of DN on the biochemical pathway leading to PKC activation seems to be equally complex. The inhibitory activity on thrombin as well as on TPA-induced protein phosphorylation would suggest the existence of at least two different targets. These could be tentatively indicated in phosphatidylserine and the phosphoinositides. The first phospholipid is an essential cofactor for PKC and DN has been shown to have a high affinity for it [44]. The inhibitory effect of DN on thrombin and DX-induced protein phosphorylation, analogous to that caused by neomycin, and on serotonin release would suggest the formation of a complex with phosphoinositides that is expected to be structurally different from that formed by DX because of the different hydrophobicity of the two drugs.

In conclusion our results indicate that the two antitumor anthracyclines DX and DN affect in several ways the membrane associated activities responsible for the transduction of external signals. If no conclusive molecular mechanism for DX and DN action is proposed here, the evidence is presented of a substantially different behaviour of DX with respect to its parent drug DN.

The concentrations of drug found in experimental tumors are lower than those used in our system, the elimination rate is, however, very slow [52]. Then tumor cells remain in contact with low doses of drug for a very long time *in vivo*. Although the biological consequences of a low level prolonged stimulation of the phosphoinositide cycle are not known precisely yet, an effect on cell proliferation and differentiation cannot be excluded. In any case, it is stimulating the finding that an antitumor drug can affect a biochemical pattern which has been shown to be impaired in transformed cells lines [21–26] and tumors [53].

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