Inhibition of Human Erythrocyte Inositol Lipid Metabolism by Adriamycin

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ABSTRACT

Incubation of human erythrocytes with Adriamycin prevented their morphological transition from discs to echinocytes when they were either depleted of ATP or loaded with calcium. This effect was dependent upon drug concentration and cell density. Adriamycin (10^-5 M) prevented, by >90%, the echinocytosis of 10^7 cells/ml (S. B. Chahwala and J. A. Hickman, Cancer Res., 45: 4986-4989, 1985), and 5 x 10^-4 M prevented that of 10^6 cells/ml. There was a poor correlation between the effects of Adriamycin as a modulator of this morphological transition and its potency as an inhibitor of calmodulin. Using inside-out red blood cell vesicles, Adriamycin inhibited calmodulin dependent Ca^2+ uptake with a 50% inhibitory concentration of 5 x 10^-4 M. Adriamycin thus differs from other amphipathic drugs, such as those of the phenothiazine class, where inhibition of calmodulin correlated well with effects on erythrocyte morphology (G. A. Nelson, M. L. Andrews, and M. J. Karnovsky, J. Cell Biol., 96: 730-735, 1983). After 12 h of ATP depletion, levels of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2] extracted from 10^8 erythrocytes/ml fell by 57% and after 48 h they fell by 97%, changes which were coincident with a 100% transition of morphology to echinocytes. Adriamycin, 5 x 10^-4 M-1 x 10^-3 M, maintained 10^6 cells/ml in a discocyte morphology and maintained PtdIns(4,5)P2 levels at 60-70% of the time zero controls, independently of the size of the fall in PtdIns(4,5)P2 levels. The data suggested that Adriamycin inhibited a discrete pool of PtdIns(4,5)P2, which may be responsible for the maintenance of a discocyte morphology. Neomycin, 10^-5 M, had no effect on the ATP depletion-induced discocyte-echinocyte transition of 10^8 erythrocytes/ml or on the fall in PtdIns(4,5)P2 levels. Adriamycin, like neomycin, prevented the calcium-induced breakdown of erythrocyte membrane vesicle PtdIns(4,5)P2 to inositol trisphosphate (50% inhibitory concentration, 7 x 10^-4 M) but, unlike neomycin (50% inhibitory concentration, 4.25 x 10^-4 M) it was able to inhibit breakdown by 100% at higher concentrations.

INTRODUCTION

The aminoglycoside antibiotic Adriamycin (doxorubicin) is a potent antitumor drug in humans. It has traditionally been classified as a DNA intercalator and more recently has been studied its effects on the erythrocyte membrane (6-8). We found Adriamycin to be a potent modulator of the morphological transition of erythrocytes from the discocyte to echinocyte form (8) (Fig. 1). Unlike other amphipathic drugs, e.g., of the phenothiazine class, which also modulate this transition, Adriamycin was able to prevent the formation of echinocytes induced by calcium loading, it had no effects on discocyte morphology per se, and its activity to maintain discocyte morphology was partly reversed by N-acetylglucosamine (8).

These findings suggested to us that Adriamycin had a novel and distinctive action on the erythrocyte membrane and that its effects were not due to a simple mechanical intercalation of the inner membrane leaflet, as has been suggested to explain the activity of other amphipaths (9).

There is considerable interest in the mechanisms whereby changes in erythrocyte morphology are controlled (10). Recent findings suggest that the maintenance of PtdIns(4,5)P2 levels in the inner leaflet of the membrane may play a pivotal role in the maintenance of a discocyte morphology (Fig. 1) (11-13). Adriamycin has a particular avidity for negatively charged phospholipids (reviewed in Ref. 14), and since another aminoglycoside antibiotic, neomycin, has been suggested to bind to PtdIns(4,5)P2 so as to prevent its metabolism, including that in the erythrocyte (11, 14-17), we have investigated the effects of Adriamycin on erythrocyte inositol lipid metabolism.

MATERIALS AND METHODS

Materials. Human blood, which was less than 1 week old, was obtained from the local blood bank or was donated by healthy colleagues and used immediately. Adriamycin, trifluoperazine, and bovine brain calmodulin were purchased from Sigma Ltd. (United Kingdom). Isotopes were purchased from Amersham international (United Kingdom). All other chemicals and reagents were of analytical grade.

Transition of Erythrocyte Morphology by ATP Depletion. Blood was washed three times in a buffer containing 10 mM KCl, 130 mM NaCl, 2 mM MgCl2, and 15 mM Tris-HCl (pH 7.4). Cells were counted by the use of a hemocytometer. From 10^10 to 10^11 cells/ml were resuspended in this buffer and incubated at 37°C for up to 48 h, with or without Adriamycin, at various concentrations. Morphology was assessed by the criteria of Bessis (18) using a light microscope fitted with Nomarski optics.

Assay of Inhibition of Calmodulin. Calmodulin deficient inside-out erythrocyte vesicles were prepared by the method of Gietzen et al. (19). The vesicles (30-50 μg protein/ml) were incubated at 37°C, with or without drugs, in a medium which contained 130 mM KCl, 2 mM MgCl2, 200 μM ethylene bis(oxyethylenenitrite)tetraacetic acid, and 190 μM CaCl2 in 20 mM imidazole-HCl (pH 7.0). 45Ca^2+ (0.1 μCi/ml) was added and the reaction started by the addition of 2 mM ATP, with or without 50 nM bovine brain calmodulin. At the times shown, 1-ml samples were filtered through a 0.45-μm Millipore glass fiber filter. The filters were washed with 5 ml of ice-cold buffer and dried, and radioactivity was counted in a Packard Tri-Carb scintillation counter.

32P Labeling of Erythrocyte Inositol Lipids. Whole blood (100 ml) was centrifuged at 3000 x g for 5 min and washed 3 times with 154 mM NaCl in 1.5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.2). The pelleted erythrocytes were then incubated for 2 h in a buffer which contained 5 mM sodium pyruvate, 1.8 mM K2HPO4, 1 mM inosine, 1 mM adenine, 10 mM glucose, 1.8 mM magnesium glutonate, 2 mM calcium glutonate, and 0.07% dilaoyzed bovine serum albumin.

The abbreviations used are: PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; InsP3, inositol trisphosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; IC50, 50% inhibitory concentration.

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Fig. 1. Diagramatic representation of the relationship proposed between erythrocyte morphology and the metabolism of phosphoinositides (based on Refs. 11 and 12). DG, diacylglycerol.

-albumin in 188 mm sodium N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.2. This treatment restored all of the cells to a discocyte morphology. [32P]phosphate (2 mCi) was added and the incubation was continued for a further 2.5 h, after which time the ATP of the cells was equilibrated with 32P (20).

Measurement of Phosphoinositides in Erythrocytes. 32P-Labeled erythrocytes (10 ml) were washed twice in a buffer of 10 mm KCl, 130 mm NaCl, and 2 mm MgCl2 in Tris-HCl (pH 7.4) and then resuspended in this buffer at 106 cells/ml. Duplicate samples of cells were either incubated for 12 or 48 h, with or without drugs, or sampled immediately to determine the levels of inositol lipids. There was no cell loss over the incubation periods used. To determine the lipid levels, the cell suspension was added to 200 ml of a lysis buffer (1 mm EDTA in 20 mm Tris-HCl, pH 7.4) and spun at 15,000 x g for 10 min and the process repeated until the pellet was white. To the pellet were added 5 ml of 20% trichloroacetic acid and the mixture was left for 20 min on ice. It was then spun at 3000 x g for 5 min, the supernatant was removed, and the pellet was washed twice in 10 ml of 5% trichloroacetic acid which contained 1 mm ethylene bis(oxyethylenenitrite)tetraacetic acid, then finally washed in 10 ml of distilled water. The lipids were extracted by the addition of 3.75 ml of chloroform:methanol:12 M hydrochloric acid (50:100:1 by volume) for 20 min; 1.25 ml chloroform and 1.25 ml 1 M hydrochloric acid were then added to separate the phases. The mixture was vortexed and spun at 3000 x g for 5 min, the upper phase was discarded, and to the lower phase was added the upper phase from chloroform:methanol:1 M hydrochloric acid (1:1:0.9 by volume). After mixing and centrifugation as before, the upper phase was discarded and 1-ml portions of the lower phase, which contained the polyphosphoinositides, were subjected to mild alkaline hydrolysis by the method of Ellis et al. (21). Analysis of the deacylated inositol phospholipids was performed by the method of Downes and Micheli (11).

Measurement of Inositol Phosphates and Phosphoinositides from Erythrocyte Vesicles. Erythrocyte vesicles were prepared by the lysis of 32P-labeled erythrocytes, prepared as described above, in 1 mm EDTA in 20 mm Tris-HCl, pH 7.4. The lysate was spun at 15,000 x g for 10 min, and the pellet was washed 4 times in this lysis solution until it was white and was then given a final wash in 20 mm Tris-HCl (pH 7.4) with no EDTA. The erythrocyte ghosts (0.2 ml, equivalent to approximately 3 mg protein/ml) were incubated for 0.5 h at 37°C, with or without drugs, before the addition of 0.5 mm Ca2+. After 10 min, the preparation was added to 8 ml of ice-cold distilled water and centrifuged at 15,000 x g for 10 min. Aliquots (7 ml) of the supernatant were removed and loaded on to anion-exchange columns of Dowex 1 (1 ml X10, 200–400 mesh, formate form). The inositol phosphates were separated by the method of Downes and Michell (11). Two-m1 fractions were mixed with 15 ml of Optiphase MP and counted in a Packard Tri-Carb scintillation counter.

Phosphoinositides were extracted and analyzed by the treatment of the pellet obtained by centrifugation at 15,000 x g for 10 min with 3.75 ml of chloroform:methanol:12 M hydrochloric acid (50:100:1) as described above for the measurement of these lipids in intact erythrocytes.

RESULTS

Fig. 2a shows that addition of calmodulin to inside-out erythrocyte vesicles which had been stripped of calmodulin stimulated the uptake of 45Ca2+. The residual uptake of 44Ca2+...
into the stripped vesicles is probably due to the presence of residual erythrocyte calmodulin. A classical inhibitor of calmodulin, trifluoperazine (1 x 10^{-3} M), completely inhibited both the calmodulin dependent and the residual, basal uptake of "Ca^{2+}. Adriamycin did not inhibit calmodulin dependent or basal "Ca^{2+} uptake into the erythrocyte vesicles until its concentration exceeded 1 x 10^{-4} M (Fig. 2) and the IC_{50} of the drug was 5 x 10^{-4} M.

The falls in the levels of PtdIns(4)P and PtdIns(4,5)P_{2} after either a 12- or a 48-h period of ATP depletion of 10^{9} erythrocytes/ml are shown in Table 1. PtdIns(4,5)P_{2} levels fell progressively over 48 h, whereas levels of Ptdlns(4)P did not change significantly after 12 h but decreased after 48 h. In each of these experiments, the conditions of ATP depletion were sufficient to induce a 100% change in morphology from discocytes to echinocytes when Adriamycin was not present. In previous experiments we found that 10^{-3} M Adriamycin maintained discocyte morphology when incubated with 10^{7} cells/ml (8). An increase in drug concentration was required as the cell density was increased to 10^{9} cells/ml. It has been reported that the cytotoxicity of Adriamycin is similarly dependent upon cell density (22). In the presence of either 5 x 10^{-4} or 1 x 10^{-3} M concentrations of the drug per 10^{9} erythrocytes per ml a complete maintenance of discocyte morphology was observed, although we sometimes observed these discocytes to have a "mottled" and slightly flattened appearance. This was also observed in a previous study (6). Concomitant with the maintenance of the discocyte morphology by Adriamycin, there was an inhibition of the fall in the levels of PtdIns(4,5)P_{2} which was associated with the formation of echinocytes (Table 1). Results from a representative experiment are shown in Fig. 3. In a series of six experiments the radioactivity associated with PtdIns(4,5)P_{2} at the commencement of the experiment was never less than 20,000 cpm. Under identical conditions, neomycin (1 x 10^{-3} M) had no effect on the morphological transition or upon the fall in levels of inositol phospholipids.

When erythrocyte membrane vesicles were treated with calcium (5 x 10^{-4} M), breakdown of the inositol lipids occurred and the water soluble inositol phosphates were released (Table 2). Neither Adriamycin nor neomycin alone caused breakdown of inositol phospholipids or release of inositol phosphates under these conditions. Incubation with either Adriamycin (Fig. 4a) or neomycin (Fig. 4b) inhibited the Ca^{2+}-induced release of both InsP_{3} and InsP_{2}. The IC_{50}s for Adriamycin were 7 x 10^{-4} M for InsP_{3} release and 8 x 10^{-4} M for InsP_{2} release. Neomycin had similar IC_{50}s under these conditions: 4.25 x 10^{-4} M for the inhibition of InsP_{3} release; and 4 x 10^{-4} M for the inhibition of InsP_{2} release. However, whereas Adriamycin was able to completely inhibit the breakdown of phosphoinositides at 10^{-4} M, the inhibition by neomycin reached a plateau (Fig. 4). These results with neomycin are almost identical to those reported by

### Table 1: Effects of Adriamycin on the falls in PtdIns(4)P and PtdIns(4,5)P_{2} induced by ATP depletion of erythrocytes

<table>
<thead>
<tr>
<th>Concentration of Adriamycin (n/10^{9} cells/ml)</th>
<th>Time of ATP depletion (h)</th>
<th>PtdIns(4)P</th>
<th>PtdIns(4,5)P_{2}</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12</td>
<td>97.5 ± 14.4</td>
<td>42.3 ± 6.2</td>
<td>4</td>
</tr>
<tr>
<td>5 x 10^{-4}</td>
<td>12</td>
<td>78.7 ± 13</td>
<td>61.4 ± 16</td>
<td>2</td>
</tr>
<tr>
<td>1 x 10^{-3}</td>
<td>48</td>
<td>79.5 ± 5.9</td>
<td>67 ± 5.2&quot;</td>
<td>3</td>
</tr>
<tr>
<td>0</td>
<td>48</td>
<td>28.8 ± 3.9</td>
<td>8.8 ± 8.1</td>
<td>2</td>
</tr>
<tr>
<td>1 x 10^{-3}</td>
<td>48</td>
<td>60.7 ± 8.9</td>
<td>51.1 ± 15&quot;</td>
<td>2</td>
</tr>
</tbody>
</table>

* Procedures detailed in "Materials and Methods."
* Significantly different from control, P = 0.05.
* Significantly different from control, P = 0.005.

Downes and Michell (11). Inhibition was measured 10 min after the addition of calcium, a time at which release of both of the inositol phosphates was almost maximal and, under conditions where magnesium was absent, a time when they underwent very slow breakdown (11). The levels of radioactivity associated with PtdIns(4,5)P_{2} were between 19,000 and 25,000 cpm.

### DISCUSSION

In a previous study of the modulation of erythrocyte morphology by Adriamycin, we suggested that the drug had a distinctive mechanism of action in comparison with other amphipathic drugs, such as those of the phenothiazine class (8). The results of the present study would seem to support our earlier conclusion regarding the mechanism of its interaction with the erythrocyte membrane. For example, in a report by Nelson et al. (23) of the activity of 39 amphiphatic compounds which maintained discocyte shape, there was an excellent correlation between their potency to modulate morphology and their potency as calmodulin inhibitors, the only exception being that of the calcium channel blocker verapamil. We have found Adriamycin to be a weak inhibitor of calmodulin, as measured by the inhibition of calmodulin dependent Ca^{2+} uptake into inside-out RBC vesicles (Fig. 2). Additional, but indirect, evidence to support this conclusion comes from other studies of ours: Adriamycin had no effects on the membrane potential of erythrocytes (24) or mouse L1210 leukemia cells (25), whereas agents which raise intracellular Ca^{2+}, by inhibition of the calmodulin dependent Ca^{2+} efflux pump, bring about a Ca^{2+} activated K^{+} efflux, which results in hyperpolarization. In Adriamycin treated L1210 cells there was no increase in intracellular calcium, measured by arsenazo-III, until 2 h after incubation with a 10^{-5} M drug concentration (26), evidence which suggests that the function of the calmodulin dependent Ca^{2+} pump was not impaired.

The importance of inositol phospholipids to the control of erythrocyte morphology has been discussed in the introduction. Fig. 3 and Table 1 show that Adriamycin inhibits the morphological transition of erythrocytes at the same time that it inhibits the breakdown of PtdIns(4,5)P_{2}. Because of the high cell density required for the analysis of these lipids, and consequently the
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Table 2 32P-Labeled inositol lipids in, and inositol phosphates released from, erythrocyte membrane vesicles PtdIns(4)P and PtdIns(4,5)P2.

<table>
<thead>
<tr>
<th>Time</th>
<th>PtdIns(4)P released</th>
<th>PtdIns(4,5)P2 released</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6,076 ± 524</td>
<td>20,176 ± 2,192</td>
</tr>
<tr>
<td>Ca2+ (0.5 mM)</td>
<td>3,148 ± 168</td>
<td>11,308 ± 596</td>
</tr>
<tr>
<td>Neomycin (1 mM)</td>
<td>6,000 ± 804</td>
<td>19,576 ± 2,212</td>
</tr>
<tr>
<td>Adriamycin (1 mM)</td>
<td>6,444 ± 1,360</td>
<td>19,892 ± 2,716</td>
</tr>
</tbody>
</table>

*Representative results, from a single experiment performed in duplicate, of the calcium-induced changes in 32P-associated radioactivity of erythrocyte membrane vesicle PtdIns(4)P and PtdIns(4,5)P2, and of the InsP3 and InsP2, released (means ± SD).

Fig. 4. Percentage inhibition (a) by Adriamycin and (b) by neomycin of the calcium stimulated release of inositol phosphates, over a 10-min period, from erythrocyte vesicles. ○, InsP2 release; ●, InsP3 release. Points, means; bars, SD, n = 3.

Fig. 4. Percentage inhibition (a) by Adriamycin and (b) by neomycin of the calcium stimulated release of inositol phosphates, over a 10-min period, from erythrocyte vesicles. ○, InsP2 release; ●, InsP3 release. Points, means; bars, SD, n = 3.

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References

Unpublished work.
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