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Inhibition by adriamycin of calmodulin-sensitive and phospholipid-sensitive calcium-dependent phosphorylation of endogenous proteins from heart

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Adriamycin, a lipid-interacting anti-cancer agent, was found to inhibit phospholipid-sensitive Ca^{2+}-dependent phosphorylation of endogenous proteins from the cytosol of the guinea-pig heart. The drug, unexpectedly, also inhibited phosphorylation of separate endogenous proteins in the cardiac cytosol and membranes catalysed by the calmodulin-sensitive species of Ca^{2+}-dependent protein kinase. In both phosphorylation systems, the inhibition by adriamycin was reversed by either phospholipid (phosphatidylserine or cardiolipin) or calmodulin respectively. Adriamycin also inhibited phosphorylation of histone (exogenous protein) catalysed by purified cardiac phospholipid-sensitive Ca^{2+}-dependent protein kinase, but not that by cyclic AMP-dependent and cyclic GMP-dependent protein kinases. It appears that Ca^{2+}-dependent protein phosphorylation systems, regulated either by phospholipid or calmodulin, may represent hitherto unrecognized sites of action of adriamycin. It remains to be seen whether inhibition by adriamycin of these systems is related to the severe cardiotoxicity, the major adverse effect of the drug that limits its clinical usefulness.

The action of adriamycin (doxorubicin hydrochloride), an anthracycline aminoglycoside antibiotic clinically used for the treatment of leukaemia and solid tumours (DiMarco *et al.*, 1969; Oldbam & Pomeroy, 1972), has been shown to inhibit DNA and RNA synthesis (Calendi *et al.*, 1965; Harteel *et al.*, 1975) through intercalation in DNA (DiMarco & Arcamone, 1975; Pigram *et al.*, 1972). Unfortunately, the clinical value of adriamycin is limited by its unusual cardiotoxicity, which is cumulative and often irreversible (Minow *et al.*, 1975; DiMarco, 1975). The cytotoxicity of the drug is probably in part related to its binding to the cell membranes (Chatelain *et al.*, 1976; Tritton *et al.*, 1978) by interacting with the negatively charged phospholipids, such as phosphatidylserine and cardiolipin (Goormaghtigh *et al.*, 1980).

Takai *et al.* (1979) have recently described a Ca^{2+}-dependent protein kinase that requires phospholipid instead of calmodulin as a cofactor. We have reported a widespread occurrence of this new Ca^{2+} target enzyme in various tissues and phyla of the animal kingdom (Kuo *et al.*, 1980). We have also shown the presence of endogenous substrate proteins for phospholipid-sensitive Ca^{2+}-dependent protein kinase, which are distinguishable from those for calmodulin-sensitive Ca^{2+}-dependent protein kinase, in the cerebral cortex (Wrenn *et al.*, 1980), heart (Katoh *et al.*, 1981) and a variety of other mammalian tissues (Wrenn *et al.*, 1981*a*). We suspected that the phospholipid-sensitive enzyme system may be affected by adriamycin, due to the ability of the drug to interact with phospholipid (Goormaghtigh *et al.*, 1980). In the present studies, we found that adriamycin inhibited not only the Ca^{2+}-dependent phosphorylation of myocardial proteins augmented by phospholipid, but also the process augmented by calmodulin.

Experimental

Materials

Phosphatidylserine (bovine brain), cardiolipin (bovine heart), lysine-rich histone (type III-S), histone (mixed, type II) and adriamycin were from Sigma (St. Louis, MO, U.S.A.).

Methods

The fresh hearts from young adult guinea pigs of both sexes were homogenized in 10 vol. of 0.25 M-sucrose in 25 mM-Tris/HCl (pH 7.5) containing 10 mM-$MgCl_2$ and 50 mM-2-mercaptoethanol, and the homogenate was centrifuged at 105000 *g* for 60 min. The resulting pellet (total particulate) was

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taken up in the original volume of homogenization solution and used directly as the source of protein kinases and their substrates (Katoh *et al.*, 1981). We noted that substrate proteins for either phospholipid-sensitive or calmodulin-sensitive Ca^{2+}-dependent protein kinase were not directly demonstrable in the cytosol (105000 *g* supernatant), unless it was further fractionated as follows (Katoh *et al.*, 1981). The fraction precipitated with $(NH_4)_2SO_4$ (35–70%, w/v) contained the phospholipid-sensitive enzyme and its substrates (Fig. 1*a*), whereas the fraction from DEAE-cellulose eluted with 100mM-$(NH_4)_2SO_4$ (in 20mM-Tris/HCl, pH 7.5) contained the calmodulin-sensitive enzyme and its substrate proteins (Fig. 1*b*). Calmodulin was eluted with a higher $(NH_4)_2SO_4$ concentration (450mM) from DEAE-cellulose in the same experiment. The methods for phosphorylation of cardiac proteins, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and subsequent autoradiography of the ^{32}P-labelled proteins were as described previously (Wrenn *et al.*, 1980), except that the concentration of [γ-^{32}P]ATP used was increased from 3.75μM to 30μM. Higher concentrations (0.3 and 1mM) of ATP were also used in some experiments; the results obtained indicated that lower ATP concentrations (30μM and lower) were best for demonstrating phospholipid-sensitive and calmodulin-sensitive Ca^{2+}-dependent phosphorylation of endogenous proteins from the heart (Katoh *et al.*, 1981) (such as shown in Fig. 1), and from the cerebral cortex and other tissues (Wrenn *et al.*, 1981*a*), as well. Calmodulin was purified to apparent homogeneity from the rat brain extract by the fluphenazine affinity method of Charbonneau & Cormier (1979). [γ-^{32}P]ATP was prepared by the method of Post & Sen (1967).

Phospholipid-sensitive Ca^{2+}-dependent protein kinase was purified over 10000-fold and to about 90% homogeneity from the bovine heart extract through the steps of $(NH_4)_2SO_4$ fractionation and

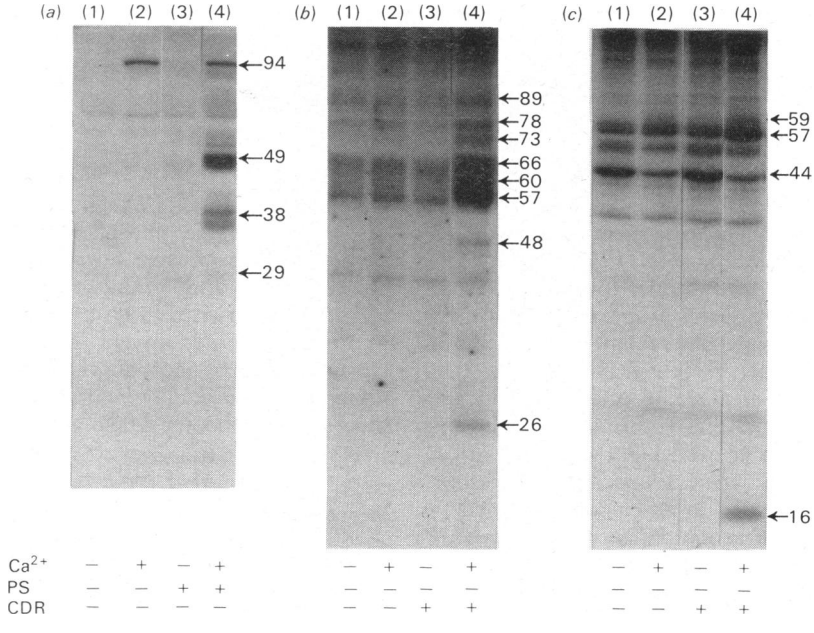

Fig. 1. *Autoradiograph showing phosphorylation of endogenous proteins in the guinea-pig heart under various incubation conditions*

The sources of endogenous protein kinases and their substrate proteins were: (*a*) the fraction precipitated from the heart cytosol with $(NH_4)_2SO_4$ (35–70%, w/v); (*b*), the fraction from the heart cytosol eluted from DEAE-cellulose with 100mM-$(NH_4)_2SO_4$; (*c*), the total, unfractionated particulate fraction of the heart. Phosphorylation of the endogenous proteins was carried out as described by Wrenn *et al.* (1980) in 0.2ml at 30°C and for 5min in the presence and absence of $CaCl_2$ (0.1μmol; final concentration, 500μM), phosphatidylserine (PS, 5μg) and calmodulin (CDR, 2μg), as indicated. EGTA (250μM) was included in all cases to minimize phosphorylation seen in the absence of added $CaCl_2$. The separating gel used was 12% (*a* and *c*) or 10% (*b*) acrylamide containing 0.1% sodium dodecylsulphate. The amount of protein applied to each well was 30μg. The molecular weights ($\times 10^{-3}$) are indicated by the bands on the gels.

DEAE-cellulose, controlled-pore glass, Sephadex G-200 and phosphatidylserine–Affigel 102 chromatographies (B. C. Wise, R. L. Raynor & J. F. Kuo, unpublished work). The Ca^{2+}-dependent protein kinase activity was assayed as we described previously using lysine-rich histone as substrate (Kuo *et al.*, 1980; Wrenn *et al.*, 1980). Cyclic AMP-dependent protein kinase and cyclic GMP-dependent protein kinase were purified from the bovine heart extracts as reported previously (Shoji *et al.*, 1977); their activities were assayed as we have decribed elsewhere, using mixed histone as substrate (Kuo & Kuo, 1976). Protein was determined by the method of Lowry *et al.* (1951).

Results

The presence in the guinea-pig heart cytosol of substrate proteins for phospholipid-sensitive and calmodulin-sensitive Ca^{2+}-dependent protein phosphorylation systems was demonstrated in Figs. 1(*a*) and 1(*b*) respectively. Substrates for the calmodulin-sensitive system were not detected in the fraction from the cytosol used in Fig. 1(*a*) (Katoh *et al.*, 1981), and those for the phospholipid-sensitive system were not detected in the fraction from the cytosol used in Fig. 1(*b*) (result not shown). Substrates for the calmodulin-sensitive system were identified in the total particulate fraction of the heart (Fig. 1*c*). As observed previously (Katoh *et al.*, 1981), no substrates for the phospholipid-sensitive system were detected in the same total particulate fraction used in Fig. 1(*c*), nor in the subcellular particulate fractions enriched in nuclei, mitochondria, microsomes or plasma membrane (results not shown).

Adriamycin inhibited, in the presence of a fixed concentration (5 μg/0.2ml) of phosphatidylserine and in a concentration-related manner, phosphorylation of at least three cytosolic substrate proteins (mol.wts. 49000, 38000 and 29000) for the phospholipid-sensitive Ca^{2+}-dependent protein kinase system (Fig. 2*a*). The inhibition by adriamycin (100 μM) seen in the same experiments was overcome by increasing concentrations of phosphatidylserine (Fig. 2*b*), indicating the competitive nature of the interaction between adriamycin and phospholipid. It has been reported that cardiolipin is a major phospholipid found in the heart (Simon & Rouser, 1969), and that adriamycin has the highest affinity for cardiolipin compared with other phospholipids (Goormaghtigh *et al.*, 1980). We noted that, as for phosphatidylserine shown in Fig. 2(*a*), cardiolipin (5 μg/0.2ml) augmented the Ca^{2+}-

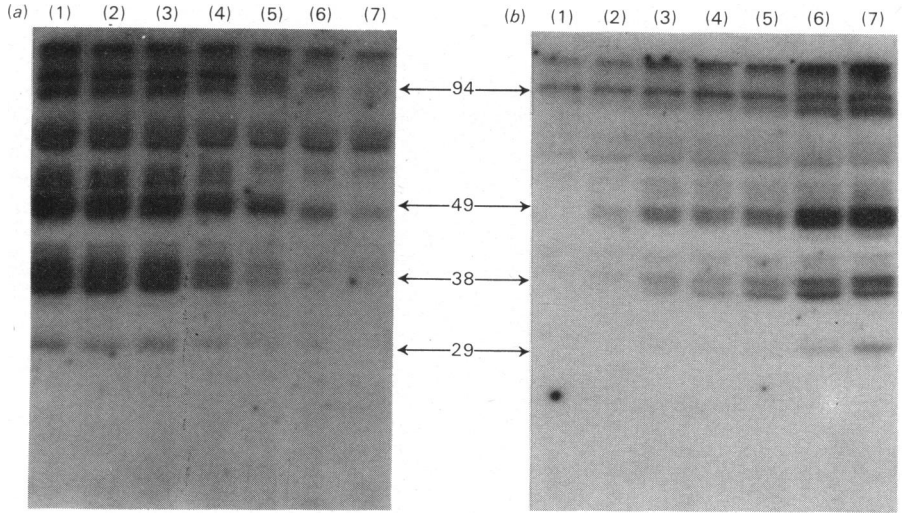

Fig. 2. *Autoradiograph showing the relationship between adriamycin and phosphatidylserine in phosphorylation of cytosolic substrates for the phospholipid-sensitive Ca^{2+}-dependent protein kinase*

The experimental conditions were as indicated in Fig. 1 with the following exceptions: (*a*), various concentrations of adriamycin (0, 10, 50, 100, 200, 500 and 1000 μM for lanes 1, 2, 3, 4, 5, 6 and 7 respectively) in the presence of a fixed concentration of phosphatidylserine (5 μg/0.2ml); (*b*), various concentrations of phosphatidylserine (0, 1, 3, 5, 10, 25 and 50 μg/0.2ml for lanes 1, 2, 3, 4, 5, 6 and 7 respectively) in the presence of a fixed concentration of adriamycin (100 μM). The cytosolic fraction used was the same as that used in Fig. 1(*a*). The molecular weights ($\times 10^{-3}$) of bands on the gel are indicated.

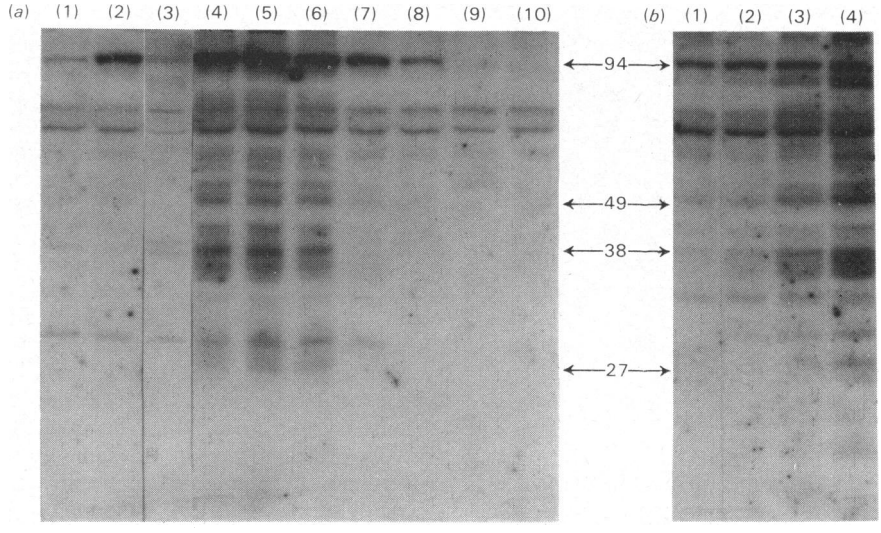

Fig. 3. *Autoradiograph showing relationship between adriamycin and cardiolipin on phosphorylation of soluble endogenous proteins in the heart*

The experimental conditions were as described in Figs. 1 and 2, except that the fraction precipitated from the heart cytosol with $(NH_4)_2SO_4$ (35–70%, w/v) was incubated under various conditions indicated below. (*a*), Lane 1, control; lane 2, with $CaCl_2$ (0.5 mM); lane 3, with cardiolipin (5 μg/0.2 ml); lane 4, with both $CaCl_2$ and cardiolipin; lanes 5, 6, 7, 8, 9 and 10, same as lane 4, but also with 10, 50, 100, 200, 500 and 1000 μM-adriamycin respectively. (*b*), All with $CaCl_2$ (0.5 mM) and adriamycin (100 μM), but also with 0, 5, 10 and 20 μg/0.2 ml of cardiolipin for lanes 1, 2, 3 and 4 respectively. The molecular weights ($\times 10^{-3}$) of bands on the gel are indicated.

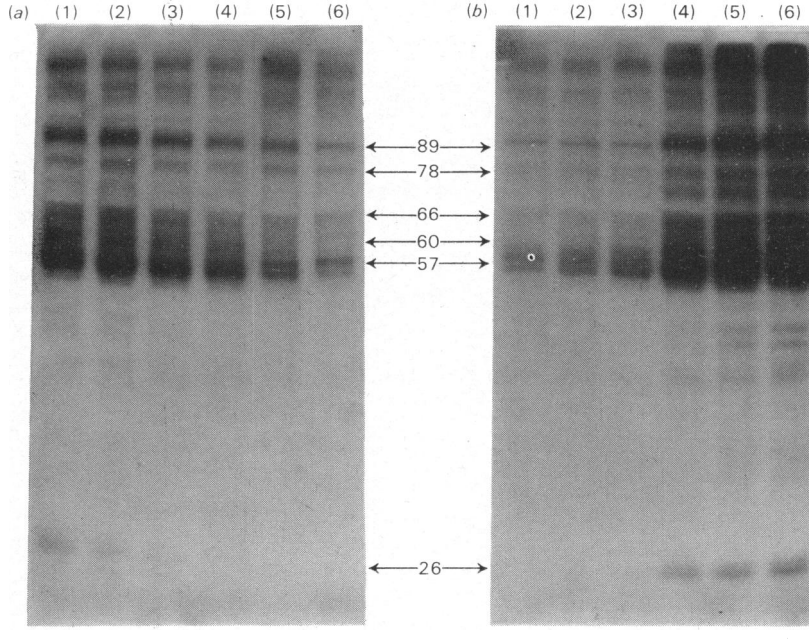

Fig. 4. *Autoradiograph showing the relationship between adriamycin and calmodulin in phosphorylation of cytosolic substrates for the calmodulin-sensitive Ca^{2+}-dependent protein kinase system*

The experimental conditions were as indicated in Fig. 1 with the following exceptions: (*a*), various concentrations of adriamycin (0, 10, 50, 100, 500 and 1000 μM for lanes 1, 2, 3, 4, 5 and 6 respectively) in the presence of a fixed concentration of calmodulin (2 μg/0.2 ml); (*b*), various concentrations of calmodulin (0, 0.4, 1, 2, 5 and 10 μg/0.2 ml for lanes 1, 2, 3, 4, 5 and 6 respectively) in the presence of a fixed concentration of adriamycin (100 μM). The cytosolic fraction used was the same as that used in Fig. 1(*b*). The molecular weights ($\times 10^{-3}$) of bands on the gel are indicated.

dependent phosphorylation of the same or similar endogenous proteins, and its effect was antagonized by increasing concentrations of adriamycin (Fig. 3*a*). Again, as for phosphatidylserine shown in Fig. 2(*b*), the inhibitory effect of adriamycin was overcome by increasing concentrations of cardiolipin (Fig. 3*b*).

It was found, quite unexpectedly, that calmodulin-sensitive Ca^{2+}-dependent phosphorylation of a number of endogenous substrates (mol.wt. 26000–89000) in the heart cytosol was also inhibited by adriamycin (Fig. 4*a*). This inhibition, moreover, was similarly overcome by increasing concentrations of calmodulin (Fig. 4*b*), suggesting that adriamycin may inhibit in a competitive manner the calmodulin-sensitive system as well.

The phosphorylation of particulate substrates (notably those of mol.wt. 57000 and 16000) for the calmodulin-sensitive system was inhibited by adriamycin to a lesser extent (Fig. 5*a*), compared with that seen for the cytosolic substrates for the same phosphorylation system shown above (Fig. 4*a*). This is probably due to binding of adriamycin to the membrane phospholipids, thus decreasing the concentration of the free and active drug that affects the calmodulin-sensitive phosphorylation system. As shown above for the cytosolic substrates (Fig. 4*b*), calmodulin was able to competitively overcome the inhibition of particulate substrate phosphorylation caused by adriamycin (Fig. 5*b*). Although results are not shown, adriamycin had little or no effect on the basal phosphorylation of cytosolic or membrane proteins seen in the absence of added $CaCl_2$, phospholipid or calmodulin.

It seems worth noting that a soluble protein of mol.wt. 94000 was phosphorylated in the presence of Ca^{2+}, and this phosphorylation was not augmented by either phosphatidylserine (Fig. 1*a*) or calmodulin (Katoh *et al.*, 1981). Furthermore, adriamycin was able to inhibit its Ca^{2+}-stimulated phosphorylation (Fig. 2*a*), and this inhibition was not overcome by either phosphatidylserine (Fig. 2*b*) or cardiolipin (Fig. 3*b*). It was also noted that Ca^{2+}, either in the presence or absence of calmodulin, inhibited phosphorylation of a particulate protein of mol.wt. 44000 (Fig. 1*c*), and that adriamycin was without effect on its phosphorylation (Fig. 5*a*). We observed in separate experiments that this protein was localized in the mitochondrial fraction (Katoh *et al.*, 1981).

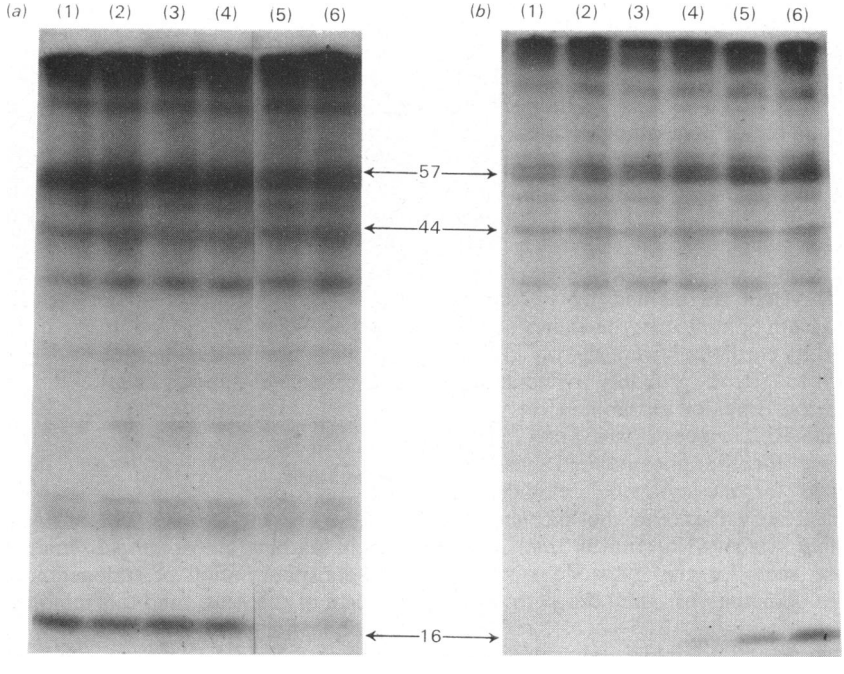

Fig. 5. *Autoradiograph showing the relationship between adriamycin and calmodulin in phosphorylation of particulate substrates for the calmodulin-sensitive Ca^{2+}-dependent protein kinase system*

The experimental conditions were as described in Fig. 4, except that the total particulate fraction (the same as that used in Fig. 1*c*) was used.

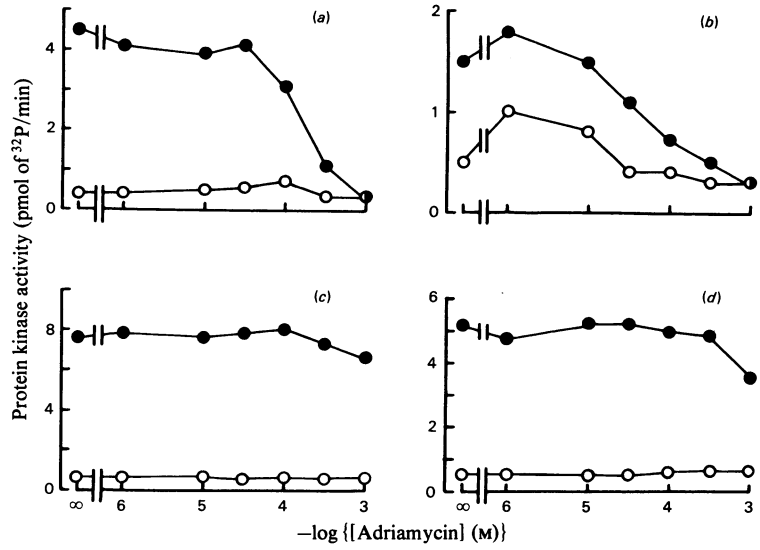

Fig. 6. *Comparative effects of adriamycin on different classes of protein kinases from the bovine heart*
The assay conditions for various protein kinases were as mentioned under 'Methods', in the presence of various concentrations of adriamycin as indicated. (*a*) Phospholipid-sensitive Ca^{2+}-dependent protein kinase (0.1 μg) was incubated in the presence of phosphatidylserine (3 μg/0.2 ml), with (●) or without (○) $CaCl_2$ (0.5 mM); (*b*) phospholipid-sensitive Ca^{2+}-dependent protein kinase (0.1 μg) was incubated in the presence of cardiolipin (5 μg/0.2 ml), with (●) or without (○) $CaCl_2$ (0.5 mM); (*c*) cyclic AMP-dependent protein kinase (10 μg) was incubated with (●) or without (○) cyclic AMP (0.5 μM); (*d*) cyclic GMP-dependent protein kinase (20 μg) was incubated with (●) or without (○) cyclic GMP (0.5 μM).

The inhibition by adriamycin of the Ca^{2+}-dependent phosphorylation of endogenous proteins augmented by either phospholipid (phosphatidylserine or cardiolipin) or calmodulin was quantified by densitometric scannings of autoradiograms such as shown in Figs. 2–5. The IC_{50} values (concentrations causing a 50% inhibition) for adriamycin were determined from the dose-dependent inhibition curves obtained for the individual substrates. The IC_{50} values for adriamycin of the cytosolic phospholipid-sensitive system augmented by phosphatidylserine ranged from 55 to 80 μM; the values of the system augmented by cardiolipin were slightly lower, ranging from 45 to 50 μM, probably reflecting a higher affinity of the drug for cardiolipin than for phosphatidylserine. IC_{50} values (50–85 μM) comparable with those for the phospholipid-sensitive system were noted for the cytosolic calmodulin-sensitive system. The values for the particulate calmodulin-sensitive system (180 and 500 μM) were higher than those seen for the cytosolic system, probably due to binding of the drug to the membrane.

Adriamycin was found to inhibit phospholipid-sensitive Ca^{2+}-dependent protein kinase purified from the bovine heart, assayed with histone as exogenous substrate and with either phosphatidylserine (Fig. 6*a*) or cardiolipin (Fig. 6*b*) as activator. The IC_{50} values for adriamycin for the above experiments were about 150 and 70 μM respectively, which were higher than the values seen for the endogenous protein phosphorylation. It is noteworthy that adriamycin slightly stimulated the basal activity (seen in the absence of added $CaCl_2$) of the enzyme augmented by cardiolipin (Fig. 6*b*) but not by phosphatidylserine (Fig. 6*a*). In comparison, adriamycin essentially had no effect on cyclic AMP-dependent protein kinase (Fig. 6*c*) and cyclic GMP-dependent protein kinase (Fig. 6*d*), both purified from the bovine heart; it slightly inhibited the cyclic nucleotide-dependent enzymes only at the highest concentration (1 mM) of the drug tested.

Discussion

The present studies demonstrated that adriamycin inhibited phospholipid-sensitive Ca^{2+}-dependent phosphorylation of endogenous proteins in the cytosol of the heart, and this inhibition was reversed by phospholipids (Figs. 2 and 3). These observations are in line with the reports that the drug binds to phospholipids (Goormaghtigh *et al.*, 1980) and to membranes (Tritton *et al.*, 1978), presumably through interactions with the lipid components. It is intriguing that adriamycin also inhibited, with a

similar effectiveness, the calmodulin-sensitive Ca^{2+}-dependent protein phosphorylation system in the heart cytosol, and, moreover, this inhibition was similarly overcome by calmodulin (Fig. 4). Since the effect of adriamycin on these two Ca^{2+}-dependent protein kinase systems was competitive with respect to phospholipid and calmodulin, the IC_{50} values for the drug could be much lower when phosphorylation of endogenous proteins, either cytosolic (Figs. 2 and 3) or particulate (Fig. 4), and histone (Fig. 6) was carried out in the presence of lower amounts of either phospholipid or calmodulin.

The mechanism of action of adriamycin on the calmodulin-sensitive Ca^{2+}-dependent protein kinase system is not clear. One possibility is that the drug, being lipophilic, has affinity for the hydrophobic regions of calmodulin, and interactions of adriamycin with these regions would result in a decreased ability of the Ca^{2+}–calmodulin complex to activate the phosphorylation system. The presence of the hydrophobic regions on calmodulin and their involvements in activation of certain enzymes requiring Ca^{2+} and calmodulin have been reported by Tanaka & Hidaka (1980). It appears that the hydrophobic regions on calmodulin may provide a tentative basis for the diverse effects of certain phospholipid-interacting agents. The phenothiazine anti-psychotic drugs were found to inhibit the Ca^{2+}- and calmodulin-requiring cyclic AMP phosphodiesterase (Weiss & Levin, 1978), as well as the phosphorylation of histone by phospholipid-sensitive Ca^{2+}-dependent protein kinase (Mori *et al.*, 1980; Schatzman *et al.*, 1981), and phosphorylation of endogenous proteins from the cerebral cortex by calmodulin-sensitive and phospholipid-sensitive Ca^{2+}-dependent protein kinase systems (Wrenn *et al.*, 1981*b*). It was also observed that palmitoylcarnitine similarly inhibited both the calmodulin-sensitive and phospholipid-sensitive Ca^{2+}-dependent phosphorylation of endogenous proteins from the heart (Katoh *et al.*, 1981).

It is noteworthy that adriamycin selectively inhibited purified phospholipid-sensitive Ca^{2+}-dependent protein kinase, with little or no effect on other classes of protein kinases activated by cyclic AMP and cyclic GMP (Fig. 6). We have observed similar phenomena for the phenothiazine anti-psychotic drugs (Schatzman *et al.*, 1981) and palmitoylcarnitine (Katoh *et al.*, 1981) It appears, therefore, that the cyclic nucleotide-dependent protein phosphorylation systems are unlikely sites of action of these drugs. It is unclear at present whether inhibition by adriamycin of the two Ca^{2+}-dependent protein kinase systems is related to the cardiomyopathy caused by the drug. The answer must await establishment of roles for phosphorylation of cardiac proteins by these systems.

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