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ROLE OF THE FAST-EXCHANGING CALCIUM COMPARTMENT IN THE EARLY CARDIOTOXICITY OF ANTHRACYCLINE ANALOGS

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Abstract—Two new anthracycline analogs, 4′-*epi*-doxorubicin and 4′-deoxydoxorubicin, were tested for their cardiotoxicity and their activity on calcium turnover in guinea pig heart. The d*f*/d*t* was used as an index of contractile force; calcium turnover was studied by means of a radioisotopic technique. 4′-*Epi*-doxorubicin was found less cardiotoxic than doxorubicin, whereas 4′-deoxydoxorubicin was found almost completely devoid of cardiotoxicity. The different cardiotoxic activity was found to be linearly correlated with the relative capacity to inhibit the fast-exchanging calcium compartment in cardiac muscle: doxorubicin > 4′-*epi*-doxorubicin > 4′-deoxydoxorubicin. This result supports that the inhibition of calcium exchange is involved in development of the early cardiotoxicity of anthracyclines.

Previous investigations have shown that doxorubicin, at a dose which induces a significant negative inotropic effect in isolated guinea pig atria, also causes impairment of cellular calcium exchangeability. Kinetic studies have indicated that this drug reduces the fast-exchanging calcium compartment. In contrast, no significant effects were found on the slow-exchanging calcium compartment or on calcium uptake in the subcellular organelles involved in the regulation of intracellular calcium concentration [1–3].

The importance of the impairment of calcium turnover as a factor responsible for the early cardiotoxicity of doxorubicin was confirmed by the observation that in the same experimental model, taurine, a calcium-mobilizing drug, partially restores the calcium turnover and prevents the negative inotropic effect of doxorubicin. In contrast, verapamil, a calcium-channel blocker, enhances the inhibitory activity of doxorubicin on contractile force and on calcium transport [4, 5].

The aim of the present investigation was to ascertain whether the inhibition of calcium transport is a general mechanism that supports early cardiotoxicity induced by anthracyclines, or is limited to doxorubicin. A comparison was made among doxorubicin, 4′-*epi*-doxorubicin and 4′-deoxydoxorubicin. These new analogs showed interesting anti-neoplastic activity and lower cardiotoxicity in animal experiments as well as in preliminary clinical trials [6–12].

MATERIALS AND METHODS

Experiments were conducted on spontaneously beating guinea pig atria isolated from animals of either sex (body weight about 500 g) as previously described [1]. The bathing solution used was a Tyrode solution at 37°, aerated with 5% CO_2 in O_2, pH 7.3, composed as follows: 8 g/l NaCl (138 mM), 0.2 g/l KCl (2.7 mM), 0.2 g/l $CaCl_2$ (1.0 mM), 1.0 g/l $NaHCO_3$ (12.8 mM), 0.05 g/l NaH_2PO_4 (0.48 mM), 1.0 g/l glucose (6.94 mM).

Contractile force was recorded with an isometric tension recording system; the d*f*/d*t* was used as an index of contractility. The organs were loaded with 1 g tension, equilibrated for 60 min, and treated with 50 μg/ml of drugs. The contractile force was measured for a further 60 min. Other preparations were equilibrated in the same conditions, then submitted to different loads ranging from 0.25 to 4.0 g as previously described [13]; the drugs were added, and after 60 min a further loading curve was recorded. The action of the drug was evaluated by comparing the curve obtained after the treatment with that obtained before the administration of the drug.

Extracellular space was determined by [^{3}H]inulin and found to be about 26%. Since no statistical difference was found among the different experimental groups (by means of Student's *t*-test), a size of 26% was used for the calculation of the ^{40}Ca and of ^{45}Ca present in the extracellular spaces.

After a 60-min equilibration as described above, the organs were submitted to simultaneous administration of the drugs at a final concentration of 50 μg/ml and of $^{45}CaCl_2$ at a concentration of 0.2 μCi/ml. After 2.5, 5, 15, 30, 45 and 60 min of incubation, atria were removed, dipped three times in an ice-cold bathing solution, blotted on filter paper, weighed and submitted to a wet ashing procedure with 1:1 HNO_3:$HClO_4$ at 220 min. The residue was dissolved in 0.1 N HCl. Aliquots were taken for the spectrofluorometric assay of calcium [14] and for the ^{45}Ca radioassay by liquid scintillation counting. Efficiency was evaluated by external standardization.

Cellular ^{40}Ca and ^{45}Ca were calculated after correction for the amount present in the extracellular spaces. The ratio between the cellular-specific

activity and that in the medium corresponds to the amount of cellular calcium exchanged after different periods of exposure to ^{45}Ca. The steady-state value is reached after 60 min of exposure to ^{45}Ca; this value represents the amount of the cellular calcium exchangeable fraction. Compartmental analysis was carried out by evaluating a curve where the difference between exchanged calcium at t_∞ and calcium exchanged at each experimental time was plotted as a function of time [1]. The amount of calcium present in each compartment was calculated from the area under the curve.

Doxorubicin, 4′-*epi*-doxorubicin, and 4′-deoxydoxorubicin were supplied by Farmitalia Carlo Erba (Milan, Italy).

RESULTS

Figure 1 shows that the contractile response to different loads increases by increasing the applied load, with a peak corresponding to a load of about 1 g, and then gradually decreases. The contractile response of the organs submitted to different loads after 1 hr exposure to 90 μM doxorubicin was significantly reduced; the peak force decreased by about 35%. 4′-*Epi*-doxorubicin at the same dose produced a smaller decrease in contractile force, with an 18% reduction in the peak force. In contrast, 4′-deoxydoxorubicin was almost completely devoid of cardiotoxicity, since the decrease in peak force was less than 3%. It should be stressed that this new analog has a transient positive inotropic effect that lasted for about 5 min immediately after its introduction in the incubation medium.

Table 1 shows that the negative inotropic action was not supported by a modification of the total tissue calcium content, whereas a reduction in the calcium exchangeable fraction was observed. This effect was statistically significant only in doxorubicin-treated preparations; 4′-*epi*-doxorubicin did not produce a significant reduction in the tested parameters; 4′-deoxydoxorubicin had almost no effect.

As previously described, the calcium exchangeable fraction can be divided into two kinetically different compartments, a fast- and a slow-exchanging calcium compartment [15]. The effects of the tested drugs on these parameters are reported in Table 2, which shows that the drugs mainly reduced the fast-exchanging calcium compartment. The inhibitory effect was statistically significant for doxorubicin and 4′-*epi*-doxorubicin, whereas 4′-deoxydoxorubicin showed only a slight effect. Doxorubicin was markedly more effective than 4′-*epi*-doxorubicin. A significant action on the slow-exchanging calcium compartment was observed only for doxorubicin.

The size of the fast-exchanging cellular calcium compartment was plotted against the percentage decrease in maximal contractile force of atrial preparations exposed to the tested anthracyclines. As indicated in Fig. 2, both parameters were found to be linearly correlated.

DISCUSSION

Several different mechanisms may be involved in the doxorubicin-associated acute cardiotoxicity. On the basis of kinetic studies, a previous investigation demonstrated that the negative inotropic effect of doxorubicin is accompanied by an impairment of cellular calcium exchangeability. In particular, it was shown that doxorubicin does not modify the total tissue calcium content, whereas it significantly reduces the calcium exchangeable fraction, particularly the fast-exchanging calcium compartment. No significant effects were found on calcium uptake in

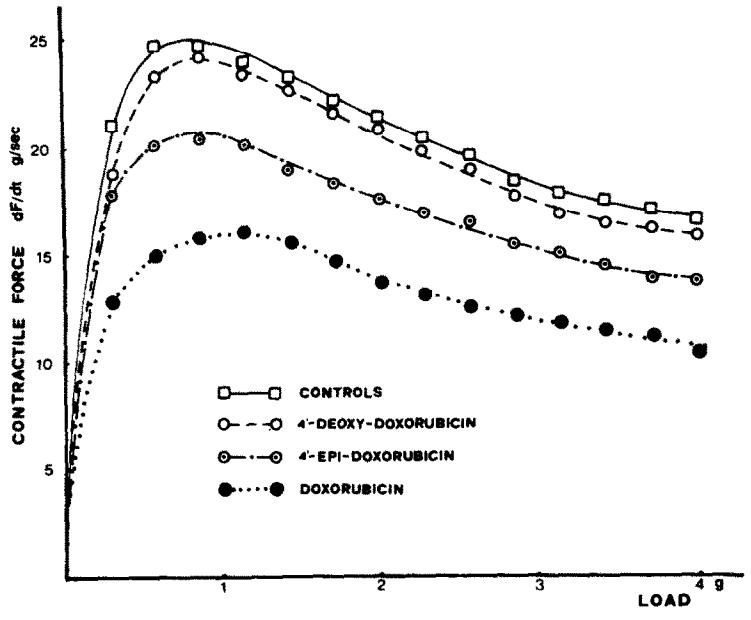

Fig. 1. Effect of 90 μM doxorubicin, 4′-*epi*-doxorubicin, and 4′-deoxydoxorubicin on contractile response to increasing loads in isolated guinea pig atria.

Table 1. Effect of 90 μM doxorubicin, 4'-*epi*-doxorubicin, and 4'-deoxydoxorubicin on cellular calcium and exchangeable calcium fraction in isolated guinea pig atria

	No. of determinations	Total calcium nEq/100 mg fresh tissue	Exchangeable calcium, %	Exchangeable calcium nEq/100 mg fresh tissue
Controls	19	329.84 ± 20.52	65.53 ± 4.01	215.07 ± 13.16
Doxorubicin	15	308.12 ± 19.97	51.55 ± 3.38*	158.83 ± 10.42*
4'-*Epi*-doxorubicin	13	322.67 ± 20.56	61.06 ± 4.41	215.51 ± 15.69
4'-Deoxydoxorubicin	15	324.25 ± 10.47	58.45 ± 3.39	189.62 ± 11.00

* $P < 0.05$.

Table 2. Effect of 90 μM doxorubicin, 4'-*epi*-doxorubicin, and 4'-deoxydoxorubicin on the fast- and slow-exchanging calcium compartment in guinea pig atria (nEq/100 mg fresh tissue)

	Fast-exchanging compartment	Slow-exchanging compartment
Controls	21.0 ± 1.2	193.9 ± 11.8
Doxorubicin	10.0 ± 0.6†	148.7 ± 9.7*
4'-*Epi*-doxorubicin	13.6 ± 0.8†	201.1 ± 13.3
4'-Deoxydoxorubicin	19.1 ± 1.1	170.4 ± 29.4

* $P < 0.05$.
† $P < 0.01$.

subcellular organelles (mitochondria and sarcoplasmic reticulum fraction) involved in the regulation of intracellular calcium concentration [2, 3].

The importance of the involvement of calcium turnover as a factor responsible for the early cardiotoxicity of doxorubicin is stressed by the observation that, in the same experimental model, taurine, a membrane calcium-mobilizing drug, partially restores calcium turnover and prevents the negative inotropic effect of doxorubicin [4]. Verapamil, a calcium-channel blocker, enhances the inhibitory activity of doxorubicin on contractile force and on calcium turnover [5].

The present investigation suggests that the inhibition of calcium transport represents a general mechanism that supports the early cardiotoxicity induced by anthracyclines and is not limited to doxorubicin. This conclusion derives from data obtained with the tested anthracycline analogs, 4'-*epi*-doxorubicin and 4'-deoxydoxorubicin. In compliance with the results of preclinical and preliminary clinical trials [11, 12], 4'-*epi*-doxorubicin was found to be less car-

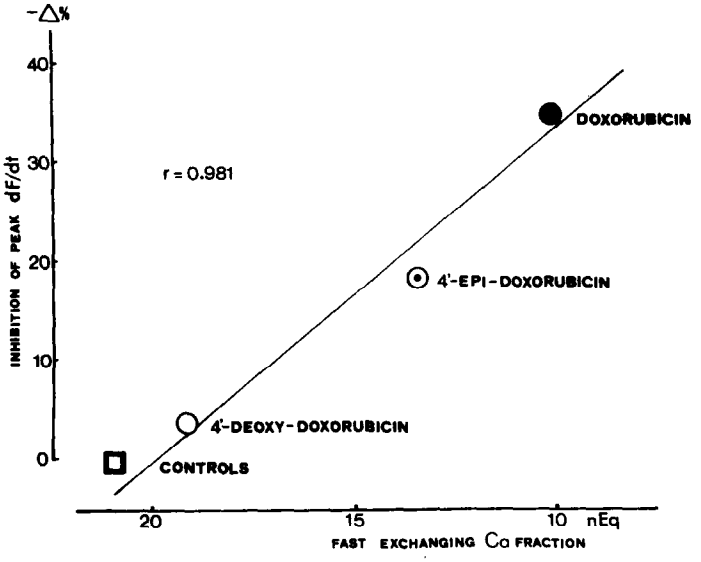

Fig. 2. Correlation between the size of the fast-exchanging calcium fraction and contractile force. The experimental points represent the mean of 7 to 8 determinations.

diotoxic than doxorubicin, and 4′-deoxydoxorubicin was found to be almost completely devoid of cardiotoxicity. These analogs influenced calcium transport in the same way as doxorubicin: in fact, they did not modify the total tissue calcium content, whereas they reduced the fast-exchanging calcium compartment. This effect was statistically significant for 4′-*epi*-doxorubicin, but not for 4′-deoxydoxorubicin. The impairment of the fast-exchanging calcium compartment was proportional to the cardiotoxic activity. In fact, this parameter and myocardial contractility were found to be linearly correlated, thus confirming the importance of the kinetically determined fast-exchanging calcium compartment.

There is strong evidence that in cardiac muscle this cellular fraction is directly correlated to the contractile properties of the muscle [15, 16]. In contrast, the slow-exchangeable fraction could hardly be related to the contractile properties of the muscle, neither does it seem to mediate conditions that cause a rapid change in contractile force. In compliance with this theory, it was recently demonstrated that cardiac glycosides produce a significant increase in the fast-exchanging calcium compartment [17].

Unfortunately, the fast-exchanging calcium compartment represents a kinetic entity that may include calcium bound to myoplasm [18], sarcoplasmic reticulum [19], and/or sarcolemma [20]. Of these structures, sarcoplasmic reticulum was unaffected by doxorubicin [3, 21], whereas myoplasm, investigated only as regards its calmodulin activity, was affected by doxorubicin [22]. The effect of doxorubicin on sarcolemmal calcium was more extensively investigated: in principle, the decrease in calcium exchangeability could be due to effects of doxorubicin brought about on unspecific calcium-binding sites. However, this possibility can be excluded, since it was demonstrated in isolated sarcolemmal vesicles that doxorubicin does not influence the calcium not specifically associated with the sarcolemma membrane [23]. In contrast, it was demonstrated that doxorubicin has the property of inhibiting the Na–Ca exchange of the sarcolemma, which is probably responsible for the calcium extrusion from myocardial cells, whereas no effect was demonstrated on the calcium-pumping ATPase or on the associated mechanism of calcium extrusion [23, 24].

In agreement with the results of the present investigation, it was observed that doxorubicin depresses calcium influx and the associated magnitude and duration of the slow action potential [25]. Both effects, inhibition of the influx and inhibition of the efflux, are of obvious importance for determining the extent of the pool of cellular calcium exchangeable fraction and are probably involved in the reduction of the calcium exchangeability induced by the drug.

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REFERENCES

1. F. Villani, F. Piccinini, P. Merelli and L. Favalli, *Biochem. Pharmac.* **27**, 985 (1978).
2. F. Villani, L. Favalli and F. Piccinini, in *Current Chemotherapy and Infectious Diseases* (Eds. C. Grassi and J. D. Nelson) Vol. 2, p. 1696. American Society of Microbiology, Washington D.C. (1980).
3. F. Villani, L. Favalli and F. Piccinini, *Tumori* **66**, 689 (1980).
4. F. Villani, F. Piccinini, L. Favalli and M. C. Chiari, *G. Ital. Chemioter.* **26**, 263 (1979).
5. L. Favalli, C. Chiari Ferrari, E. Monti, F. Piccinini and F. Vallani, *I.R.C.S. Med. Sci.* **9**, 397 (1981).
6. A. M. Casazza, A. Di Marco, C. Bertazzoli, F. Formelli, F. Giuliani and G. Pratesi, in *Current Chemotherapy* (Eds. W. Siegenthaler and R. Luthy) p. 1257. American Society of Microbiology, Washington, D.C. (1978).
7. A. M. Casazza, A. Di Marco, G. Bonadonna, V. Bonfante, C. Bertazzoli, A. Bellini, G. Pratesi, L. Sala and L. Ballerini, in *Anthracyclines: Current Status and New Developments* (Eds. S. T. Crooke and S. D. Reich) p. 403. Academic Press, New York (1980).
8. A. Di Marco, A. M. Casazza, T. Dasdia, A. Necco, G. Pratesi, P. Rivolta, A. Velcich, A. Zaccara and F. Zunino, *Chem. Biol. Interact.* **19**, 291 (1977).
9. A. M. Casazza, G. Savi and G. Pratesi, in *Current Chemotherapy and Immunotherapy* (Eds. P. Periti, and G. Gialdroni Grassi) Vol. 2, p. 1433. American Society of Microbiology, Washington, D.C. (1982).
10. A. M. Casazza, O. Bellini, G. Savi and A. Di Marco, *Proc. Am. Assoc. Cancer Res.* **22**, 267 (1981).
11. F. Villani, R. Comazzi, G. Lacaita, V. Genitoni and A. Guindani, *Int. J. clin. Pharmac.* **21**, 203.
12. V. Bonfante, F. Villani and G. Bonadonna, *Tumori* **68**, 105 (1982).
13. L. Favalli, C. Chiari, E. Monti, F. Piccinini, F. Villani, in *Current Chemotherapy and Immunotherapy* (Eds. P. Periti and G. Gialdroni Grassi) Vol. 2, p. 1449. American Society of Microbiology, Washington, D.C. (1982).
14. H. M. Von Hattinberg, W. Klaus and H. Lüllmann, *Experientia* **22**, 553 (1966).
15. G. O. Carrier, H. Lüllmann, L. Neubauer and T. Peters, *J. Mol. Cell. Cardiol.* **6**, 333 (1974).
16. F. Villani, F. Piccinini and L. Favalli, *Experientia* **32**, 1317 (1976).
17. P. C. Maitland, S. V. Lamont and G. J. Barritt, *Biochem. Pharmac.* **31**, 2471 (1982).
18. D. G. Allen and J. R. Blinks, *Nature* **273**, 509 (1978).
19. A. Fabiato and F. Fabiato, *Ann. Rev. Physiol.* **41**, 473 (1979).
20. K. D. Philipson and G. A. Langer, *J. Molec. Cell. Cardiol.* **11**, 857 (1979).
21. L. Moore, E. J. Landon and D. A. Cooney, *Biochem. Med.* **18**, 131 (1977).
22. N. Katoh, B. C. Wise, R. W. Wrenn and J. F. Kuo, *Biochem. J.* **198**, 199 (1981).
23. P. Caroni, F. Villani and E. Carafoli, *FEBS Lett.* **130**, 184 (1981).
24. P. Caroni, F. Villani and E. Carafoli, in *Advances in Studies on Heart Metabolism* (Eds. C. M. Caldarera and P. Harris) p. 35. Clueb, Bologna (1982).
25. J. Azuma, N. Sperelakis, H. Hasegawa, T. Tanimoto, S. Vogel, K. Ogura, N. Awata, A. Sawamura, H. Harada, T. Ishiyama, Y. Morita, and Y. Yamamura, *J. Molec. Cell. Cardiol.* **13**, 381 (1981).