Biochemical Pharmacology, Vol. 34, No. 11, pp. 1957–1961, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

CHARACTERIZATION OF DIGOXIN BINDING AND DAUNORUBICIN UPTAKE BY ISOLATED MATURE RAT CARDIAC MYOCYTES

N. E. CAPPS,* S. NIGDIKAR, J. H. BURNS,† E. J. WALKER‡ and J. W. DOW§
Departments of Biochemistry and Medical Cardiology, University of Glasgow, Glasgow G12 8QQ, Scotland

(*Received* 7 *September* 1984; *accepted* 18 *December* 1984)

Abstract—Myocytes isolated from ventricular muscle of mature rat heart have been used for characterization of digoxin binding and to establish whether a relationship exists between digoxin binding and uptake of daunorubicin. High- and low-affinity digoxin binding sites have been identified; respectively, $0.9 \pm 0.1 \times 10^7$ sites/myocyte, K_d 70–77 nM and $7 \pm 2 \times 10^7$ sites/myocyte, K_d 1.4–1.7 μM. Myocytes accumulate daunorubicin to an intracellular concentration 30–40 times that in the medium. We find no evidence that saturation of digoxin binding sites alters daunorubicin uptake or that daunorubicin influences binding of digoxin. Alteration of sarcolemmal membrane properties is demonstrated by inhibition of amino acid transport reflected in protein synthesis rates. Calmodulin activation of phosphodiesterase appears insensitive to daunorubicin.

Daunorubicin (DNR) and the related anthracycline compound adriamycin (ADR) or doxorubicin, are powerful anti-cancer drugs the clinical value of which is limited by cardiotoxic side effects [1–6]. Cytotoxicity in neoplastic tissue arises from intercalation of the drug between adjacent base pairs of nuclear DNA and consequential inhibition of cell division [7–13]. Cell division does not normally occur in mature myocardium and, in addition, cardiotoxicity is manifest on a time scale too brief to be accounted for by this mechanism [14].

The cardiotoxicity of anthracyclines is associated with extensive structural degeneration, including mitochondrial swelling and myofibrillar fragmentation in both man and animals [15–17]. Many biochemical abnormalities have been seen in DNR- and ADR-damaged hearts; some of the changes, such as loss of myocardial lactate dehydrogenase and creatine phosphokinase [18] and abnormal calcium movements [19–21], being symptomatic of generalized tissue damage. Generation of free radicals by the anthracyclines has been demonstrated in rat myocardial fractions [22, 23]. Limited cardiac capacity for detoxification of oxygen radicals [22, 24, 25] may account for anthracycline cardiotoxicity, although doubts about the validity of such a mechanism have been raised [26]. Depletion of reduced glutathione (GSH) has been reported by some authors [27–29] but not confirmed by Aversano and Boor [17]. Reported abnormalities of mitochondrial function include inhibition of succinic dehydrogenase [30] and cytochrome-c oxidase [31] and inhibition of coenzyme Q_{10} biosynthesis [32]. Doxorubicin reportedly binds with high affinity to the mitochondrial phospholipid, cardiolipin [33], which would account for inhibition of cytochrome oxidase [34]. Despite the many abnormalities observed, no specific biochemical lesion has as yet been confirmed as causative of the anthracycline-induced failure of myocardial contractility.

We have previously demonstrated that administration of digoxin to rats protects mechanical function when the excised hearts are subsequently perfused with DNR [14]. If hearts from untreated animals were perfused with both DNR and digoxin no protection was seen. Contractile failure in these hearts was associated with conservation of myocardial high energy compounds, ATP and creatine phosphate [14]. Whether digoxin prevents DNR binding has not been resolved, for digoxin was reported to prevent DNR binding in rat hearts *in vivo* [35], but was without influence on DNR binding to isolated perfused cat heart [36].

In this study we have used single myocytes isolated from mature rat heart in an attempt to identify a relationship between digoxin binding and DNR uptake. We report that digoxin binds to two classes of sites on isolated myocytes: high- and low-affinity sites which are characterized in this study. Saturation of these sites does not alter DNR uptake. Both classes of digoxin binding sites remain accessible in DNR-loaded myocytes. In addition we report that DNR inhibits amino acid transport but does not influence calmodulin activation of phosphodiesterase.

MATERIALS AND METHODS

Digoxin, activator-deficient phosphodiesterase and calmodulin were obtained from Sigma Chemical Co.

* Present address: Biochemistry Department, Victoria Infirmary, Glasgow, Scotland.
† Present address: Beaston Institute for Cancer Research, University of Glasgow, Glasgow, Scotland.
‡ Present address: John Curtin School of Medical Research, The Australian National University, PO Box 4, Canberra, A.C.T. 2600, Australia.
§ To whom correspondence should be addressed.

(Poole, Dorset). Digitoxose and digoxigenin were obtained from Aldrich Chemical Co. (Gillingham, U.K.). [G-^{3}H]Daunorubicin (1.32 Ci/mmole) was obtained from New England Nuclear (Boston, MA) and all other isotopes from Amersham International (Amersham, U.K.). The remaining reagents were all of analytical reagent grade. Myocytes were prepared from ventricular muscle of mature Wistar rat heart [37, 38] and maintained under aerobic conditions in medium previously described in detail [37, 39, 40]. Digoxin binding was measured in myocytes incubated at 37° in medium containing 20–800 nM [G-^{3}H]digoxin (0.2–7 μCi/ml) for 30 min. Preliminary experiments established that, for all digoxin concentrations used, digoxin binding sites are saturated within 20 min under these conditions. Digoxin binding to non-specific sites, at low digoxin concentrations (less than 150 nM), was identified by addition of 10 μM unlabelled digoxin. Because of the low solubility of digoxin in myocyte incubation medium, it was not possible to distinguish accurately specific from non-specific sites at digoxin concentrations associated with low affinity sites. Daunorubicin uptake was measured after incubating myocytes for 30 min at 37° in medium containing [G-^{3}H]DNR 10 nM–100 μM (0.5 μCi/ml). Separation of myocytes from the incubation medium was achieved by centrifuging myocytes through bromodecane: bromododecane as described by Walker *et al.* [39]. Amino acid transport and protein synthesis in myocytes were measured by methods previously described [39]. Phosphodiesterase activity was measured at 37° by the method of Itano *et al.* [41]. Results are expressed as mean ± S.D. where appropriate.

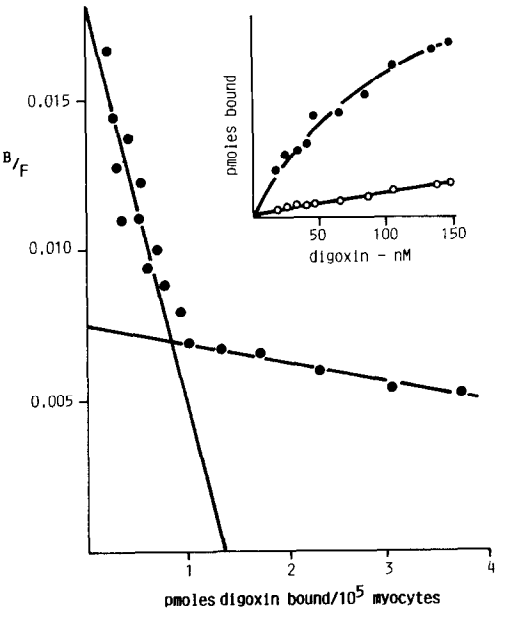

Fig. 1. Scatchard plot of digoxin binding to isolated cardiac myocytes. Myocytes were incubated at 37° for 30 min with [G-^{3}H]digoxin, 20–800 nM. The data are representative of those obtained from 6 separate myocyte preparations. The insert shows the relationship between digoxin concentrations and amount bound for total (●) and non-specific (○) binding sites.

RESULTS AND DISCUSSION

Digoxin binding sites

Figure 1 is a Scatchard plot of digoxin binding to isolated cardiac myocytes. The data indicates the presence of high- and low-affinity binding sites. High affinity sites number 0.9 ± 0.1 × 10^7 sites/myocyte, with a dissociation constant, K_d, of 70–77 nM measured in six separate myocyte preparations. Low affinity sites number 7 ± 2 × 10^7 sites/myocyte, K_d 1.4–1.7 μM. Myers *et al.* [42] report 1.1 × 10^7 sites/myocyte for rabbit myocytes. The dissociation constant we find for high affinity sites is similar to the value of 0.12 μM reported by Adams *et al.* [43] for ouabain binding to isolated rat cardiac myocytes. Onji and Lui [44] report 7.4 × 10^5 ouabain binding sites/rat cardiac myocyte, but this result is based upon a protein content per myocyte of 0.909 ng. We find 6 ng protein/myocyte [39, 45] and based upon the latter figure, the data of Onji and Lui [44] would give a maximum of 0.5 × 10^7 sites/myocyte.

In an attempt to characterize the binding configuration of digoxin to the sarcolemmal receptor, binding studies were repeated in the presence of digitoxose and digoxigenin, the sugar and steroid fragments of digoxin. Myocytes were preincubated for 30 min with one or both of these fragments at concentrations between 100 nM and 5 μM. Neither the number of digoxin binding sites nor the dissociation constants for either low- or high-affinity binding sites were altered in the presence of digitoxose, digoxigenin or both. Since the binding of these fragments to the receptor site fails to block the binding of digoxin, it seems likely that the three-dimensional structure of digoxin is dictated at least partly by the steroid–sugar bond and that the intact molecule is required for interaction with the binding site.

Uptake of daunorubicin

Preliminary experiments indicated that within the concentration range used, uptake of DNR was complete within 20 min. Intracellular concentration of DNR was calculated assuming an average myocyte water content of 25 pl/myocyte [45]. Myocytes concentrate DNR from the medium, achieving 30–40 fold greater concentrations in the intracellular fluid (Fig. 2). Thus myocytes incubated in 38 μM DNR accumulate the drug to an apparent intracellular concentration of 1.5 mM, in agreement with our previous report of DNR accumulation in isolated working rat hearts [14]. Myocytes loaded with DNR develop a bright red colour, the drug being tightly bound and exhibiting little tendency to elute from myocytes subsequently incubated in DNR-free medium. We have not yet identified intracellular sites to which DNR binds with such avidity, but cardiac myocytes are rich in mitochondria so that cardiolipin or other mitochondria phospholipids might provide sufficient binding sites [33] to account for the intracellular accumulation of DNR we observe. Binding of digoxin to myocytes was not influenced by preloading myocytes with DNR. Uptake of DNR was not altered in myocytes with

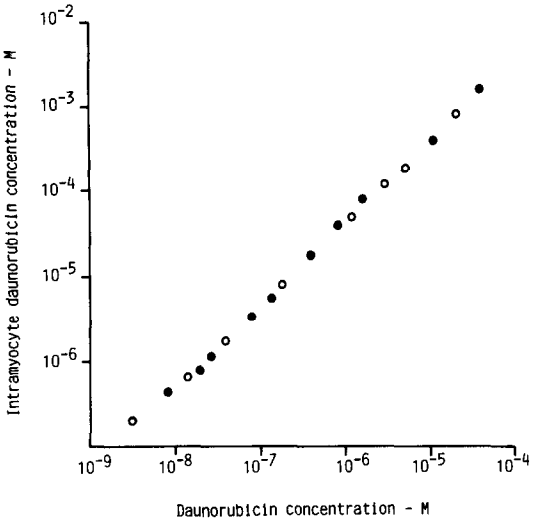

Fig. 2. Uptake of [G-^{3}H]daunorubicin by isolated cardiac myocytes. Myocytes were incubated at 37° with 10^{-9}–10^{-4} M DNR. The symbols, ○ and ●, are data obtained from two separate myocytes preparations.

the digoxin-binding sites saturated and subsequent incubation with digoxin did not stimulate release of DNR from myocytes. Thus binding of digoxin and uptake of DNR appear to be independent and we find no evidence that digoxin protection against DNR cardiotoxicity [14, 46] is mediated by changes in DNR uptake or retention.

Calmodulin-dependent processes

Interaction of ADR with calmodulin has been suggested by the results of a study in which phosphorylation of membrane proteins by calmodulin-sensitive calcium-dependent protein kinase was inhibited by ADR [47]. We have investigated the influence of DNR (10^{-9}–10^{-4} M) on calmodulin-activated phosphodiesterase activity and find neither phosphodiesterase- nor calmodulin-activation of phosphodiesterase is influenced by DNR in this concentration range.

Influence of daunorubicin on transport of amino acids and protein synthesis.

Generalized disintegration of cardiac myocyte structure, including loss of myofibrillar mass, has been reported both clinically and in experimental animals treated with DNR [15, 16, 48, 49]. Whilst inhibition of protein synthesis arising from intercalation of DNR with myocyte DNA could account for these degenerative changes, the possibilities that DNR accelerates catabolism of myocyte protein or directly inhibits protein synthesis have not been excluded.

In order to establish whether there is a short-term direct influence on protein synthesis in DNR-treated myocytes, both amino acid uptake and protein synthesis rates were measured. Myocytes were incubated with 20 or 40 μM DNR and [4,5-^{3}H]leucine (5μCi/ml) and protein synthesis rates determined by methods reported previously [39]. The rate of incorporation of leucine into total myocyte protein, measured for periods up to 3 hr, was reduced by 50% in the presence of 40 μM DNR and by 38% in the presence of 20 μM DNR. Preincubation of myocytes with DNR did not alter the extent of inhibition, which was seen within 10 min, the time of the first measurement.

Since protein synthesis in these experiments is a measure of the incorporation of extracellular leucine into myocyte protein, alterations in rate could be due to altered uptake of leucine. The influence of DNR on uptake of both leucine and the amino acid analogue, 2-aminoisobutyric acid (AIBA), was determined by methods previously described [39].

Myocytes were incubated with 20 or 40 μM DNR and either leucine or AIBA at concentrations between 0.1 and 100 μM. [1-^{14}C]-2-Aminoisobutyric acid or [4,5-^{3}H]leucine were added at 0.3 μCi/ml or 5 μCi/ml, respectively, and rates of amino acid uptake determined. The kinetic characteristics of transport of AIBA by isolated cardiac myocytes have been reported [39]. Daunorubicin inhibited uptake of both AIBA and leucine at all concentrations. Figure 3 shows the influence of 20 and 40 μM DNR on the time course of uptake of 100 μM AIBA. Similar profiles were obtained with leucine except that saturation of uptake occurs more rapidly with leucine. Daunorubicin did not alter the time course for either amino acid. This inhibition of amino acid uptake was not alleviated by pretreatment with digoxin.

Inhibition of protein synthesis has been reported for cultured chick embryo cardiac myocytes exposed to 2 μM ADR for 24 hr [50]. Inhibition of protein synthesis measured over this longer period, and in immature actively dividing cells, may reflect DNR inhibition of DNA synthesis. In our study, by comparison, the short-term inhibition of protein synthesis in non-dividing cells probably reflects inhibition of amino acid uptake. Cardiac myocytes have substantial reserves of amino acids [39], so that data

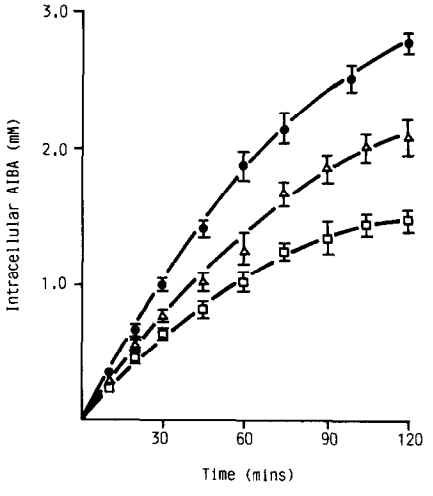

Fig. 3. Transport of 100 μM 2-aminoisobutyric acid into isolated cardiac myocytes. Control (●); 20 μM DNR (△); 40 μM DNR (□). Bar lines are S.D. for data obtained from four separate myocyte preparations.

establishing the extent to which protein synthesis depends upon extracellular rather than intracellular supplies of amino acids would be required for assessment of the impact of inhibition of amino acid transport upon protein synthesis. However, our data indicate DNR has a profound influence on the transport process within a period consistent with the onset of contractile failure [14]. If other sarcolemmal or mitochondrial membrane transport processes are similarly affected there may be early and generalized failure to maintain cellular and subcellular ion and metabolite gradients in hearts exposed to DNR. Data consistent with direct reduction by ADR of transient inward currents or indirect reduction via sodium–calcium exchange, is reported for purkinje fibres [51].

Previously we have demonstrated that digoxin protects mechanical function of isolated rat hearts against DNR-induced failure [14]. The explanation for this digoxin protection cannot be ascribed to digoxin inhibition of DNR uptake or to alleviation of the DNR-induced inhibition of amino acid uptake. Neither does DNR block digoxin binding sites on the sarcolemmal membrane. Revis and Marusic [52] have demonstrated that digoxin does not influence the doxorubicin induced increase in calcium uptake by cardiac mitochondria and that doxorubicin does not influence digoxin inhibition of Na^+/K^+ ATPase activity. Measurements of calcium exchangeability in guinea pig atria have revealed that the negative inotropic effect of ADR might be due to stabilization of the membrane-calcium complex of the fast calcium exchange process [21] which would be expected to be reversed by digoxin. In this study we have demonstrated that DNR inhibits membrane transport of amino acids, and other evidence points to DNR binding to membrane phospholipids [33]. Taken together these studies point to widespread disturbance of membrane transport and other membrane functions in hearts exposed to anthracyclines. There is yet no direct evidence that digoxin protection is mediated through counteraction of any of the early biochemical or physiological changes seen in DNR-toxic hearts.

Acknowledgements—This study was supported by grants from the British Heart Foundation and the West of Scotland Chest and Heart Association.

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