Muscle contraction on the molecular level. Actin-myosin interaction studied in an in vitro motility assay

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Acute effect of doxorubicin on the actin-myosin interaction *in vitro*

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ABSTRACT

Doxorubicin is a highly efficient anti-cancer drug with some serious cardiotoxic effects. To elucidate more details about the direct action on the actin-myosin interaction we used an in vitro motility assay.

Thin filaments were reconstituted from skeletal actin filaments, troponin, and tropomyosin. Calcium regulates the number and speed of thin filaments moving.

Doxorubicin showed an increase of speed and percentage of motile actin filaments moving over rat heart HMM molecules, suggesting a direct effect on the actin-myosin interaction. It also increased speed and percentage of motile reconstituted thin filaments moving over rabbit skeletal HMM, suggesting a specific interaction with the calcium-mediated troponin-tropomyosin complex. The irreversibility of the experiments showed a damaging effect of doxorubicin to the proteins.

Doxorubicin can be a useful tool in determining molecular details concerning calcium regulation and possibly also concerning drug-binding sites.
INTRODUCTION

Anthracyclines are highly effective anti-cancer drugs, which are used in the treatment of acute leukaemia, non-Hodgkin lymphomas, breast cancer, Hodgkin’s disease, and sarcomas [Young et al., 1981]. However, the clinical use of anthracyclines as cytostatic agents is limited by the frequent development of a dose-dependent chronic cardiomyopathy [Doroshow, 1991]. Of the clinically accepted anthracyclines, doxorubicin is the most commonly used but it is also the most cardiotoxic one [Jain et al., 1985; Cersosimo, 1992]. The anti-tumour activity of doxorubicin is understood to be the result of inhibition of nucleic acid and protein synthesis. The mechanism underlying the cardiotoxicity is however still unclear. Several hypotheses to explain the cardiomyopathy have been proposed. These include anthracycline-induced free radical formation [Doroshow, 1983], impaired myocardial calcium homeostasis through alteration of the function of cardiac sarcoplasmic reticulum (SR) [Holmberg and Williams, 1990; Pessah et al., 1990], and the formation of a highly toxic doxorubicin-metabolite, doxorubicinol [Olson et al., 1988]. Life-threatening chronic damage often develops only after several weeks or months of treatment, and sometimes even after therapy has already been completed. The most threatening chronic effect is the insidious onset of a strong cardiomyopathy, which often leads to congestive heart failure [Singal et al., 1987].

Various deleterious effects of doxorubicin on sarcolemma, intracellular membranes (such as SR), and contractile proteins have already been reported [Doroshow et al., 1985]. Doxorubicin selectively inhibits muscle specific gene expression [Kurabayashi et al., 1993]. It selectively decreases the level of mRNA of the sarcomeric genes, alpha-actin, troponin I, and myosin light chain 2, as well as the muscle-specific, but non-sarcomeric M-isoform of creatine kinase. Doxorubicin did not affect non-muscle gene transcripts (pyruvate kinase, ferritin heavy chain, and beta-actin) and its effect on muscle gene expression is limited to cardiac muscle [Ito et al., 1990]. This may explain the myofibrillar loss that characterises doxorubicin induced cardiac injury.

The acute cardiovascular effects develop within minutes or hours after the intravenous administration of the drug and are clinically characterised by hypotension, tachycardia and various arrhythmias [Singal et al., 1987].

To obtain insight into the acute effect of doxorubicin on myocardial contraction, a variety of isolated muscle preparations have been studied over the past years. Exposure of
intact heart muscle preparations to doxorubicin has been reported to produce positive inotropic effects in relatively low concentrations (~1 nM) and negative inotropic effects in higher concentrations (~1 mM) in chick heart [Azuma et al., 1981] and in rat papillary muscle [Singal et al., 1987]. A concentration-dependent increase of the contractile force by doxorubicin is reported in rabbit papillary muscle [vanBoxtel et al., 1978] and in guinea pig heart muscle [Kim et al., 1980]. In contrast, Höfling and Bolte [1981], Politi et al. [1985], Singal and Pierce [1986] and Voest et al. [1994] reported for rat and guinea pig papillary muscle, guinea pig atria, rat papillary muscle, and mice atria respectively that doxorubicin produces only negative inotropic effects. This effect was not prevented by an increase in the extracellular Ca$^{2+}$ in in vitro rat papillary muscle preparations. Therefore, a reduced supply of intracellular Ca$^{2+}$ is not a likely cause of the observed depression of contractile force [for review see Singal et al., 1987].

These conflicting data with regard to the acute contractile effects of doxorubicin on isolated muscle preparations may result, at least partially, from differences in drug dosage, the use of different myocardial preparations, the use of different animal species, and differences in the experimental protocol. Most research on the inotropic effect of anthracyclines is done in electrically stimulated intact isolated muscle preparations, providing information on the final effect of anthracyclines on the generation of force. In these models the sarcolemma and the membrane of the SR are intact. This makes it difficult to separate membrane-related effects from direct effects on the actin-myosin interaction. Experiments on skinned muscle fibre preparations with both outer and inner membranes permeabilised, showed that anthracyclines have a direct effect on the actin-myosin contractile system [deBeer et al., 1992]. Doxorubicin induced an increase in tension, which was dependent on the calcium concentration and time of incubation. This effect is dose- and anthracycline structure dependent. A small but significant right-sided shift of the calcium sensitivity curve, the relation between normalised tension and the negative logarithm of $[\text{Ca}^{2+}]$, the pCa, was observed as well. However, this small increase in calcium sensitivity is not likely to contribute to the observed increase in maximal tension. It is likely that the positive inotropic effect of doxorubicin is exerted by direct action on the force generating filaments, i.e. actin and/or myosin (possibly an effect on myosin ATPase activity), rather than being mediated by alteration of the calcium sensitivity of the troponin-tropomyosin complex of the thin filament [deBeer et al., 1992; Bottone et al., 1997].
In this chapter the direct effect of doxorubicin on actin-myosin interaction is studied in an in vitro motility assay. In this assay myosin molecules or their subfragments are attached to a nitrocellulose-coated cover slip and labelled actin filaments are moving over this myosin layer in presence of ATP. Also thin filaments can be reconstituted from actin, troponin and tropomyosin thereby restoring the troponin-tropomyosin mediated calcium regulation as originally present in muscle fibres. The effect of doxorubicin is studied on actin and thin filaments moving over rabbit skeletal HMM molecules and on actin filaments moving over rat heart HMM molecules. The species difference was imposed due to isolation problems, but turned out to be convenient for comparison with literature.

With the elimination of membrane-related events the drug has direct access to the contractile proteins thereby excluding diffusion limitations, which may interfere with processes involving muscle contraction in (permeabilised) muscle fibre experiments.

MATERIALS AND METHODS

Protein preparations

Animals

For experiments with skeletal muscle myosin and actin, a male New Zealand White rabbit, weighing 2.2 kg was used. The rabbit was anaesthetised with Hypnorm (1 ml/kg, 0.32 mg/ml fentanyl citrate and 10 mg/ml fluanisone, intramuscular, Janssen Pharmaceutics, Tilburg, The Netherlands) and the M. erector spinae was excised. For experiments with cardiac muscle myosin, five 6-week-old male Wistar rats were decapitated. Hearts were excised. In both cases animal care was in accordance with institutional guidelines.

Myosin and heavy meromyosin (HMM)

Skeletal muscle myosin was prepared according to the method of Margossian and Lowey [1982] and cardiac muscle myosin was prepared according to the method described by Sata et al [1993]. Heavy meromyosin (HMM) was prepared by chymotryptic digestion of myosin according to Kron et al [1991]. Myosin was stored at -25 °C in 50% glycerol or quickly frozen in liquid nitrogen and stored at -80 °C. HMM was stored at -80 °C after quick freezing in liquid nitrogen. Before use in the in vitro motility assay
ATP-insensitive heads were removed from aliquots of HMM as described by Kron et al. [1991]. Therefore filamentous actin (F-actin) was added to HMM in a 1:5 molar ratio and the aliquot was centrifuged with 1 to 2 mM ATP for 10 minutes at 167,000 x g in a Beckman Airfuge at 4 °C. This myosin preparation could be used in the in vitro motility assay for up to 4 hours.

Protein concentrations were measured by absorption spectroscopy or by protein assay (Bio-Rad). The molecular masses and extinction coefficients used to assay myosin and HMM concentration were 520 kDa and 0.55 (mg/ml)^−1 cm^1, and 260 kDa and 0.60 (mg/ml)^−1 cm^1 respectively, both at 280 nm. ATPase activities were measured in solution under comparable conditions as in the in vitro motility assay using a malachite green assay according to Kodama et al. [1986]. Myosin, HMM, and all other protein preparations were also analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Actin, tropomyosin and troponin**

Globular actin (G-actin) was prepared according to the procedure of Pardee and Spudich [1982] and stored at -80 °C after quick freezing in liquid nitrogen. In presence of KCl (50 mM) and ATP (1 mM) G-actin was polymerised during 3 hours on ice.

For visualisation by fluorescence microscopy, F-actin (2 μM) was labelled with 4 μM RhPh (rhodamine-phalloidin) [Kron et al., 1991] in AB-buffer (see Solutions). Reconstitution of thin filaments with (labelled) actin, tropomyosin and troponin was done by mixing the proteins at concentrations of respectively 400, 100 and 80 nM in a modified regulated filament buffer [Gordon et al., 1997] with the following composition: 98.5 mM KCl, 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS; Sigma Chemical Co., St. Louis, MO), 6 mM MgCl₂, 1 mM ethylene diamine tetra acetic acid (EDTA) and 5 mM dithiothreitol (DTT), pH 7.4 and ionic strength 125 mM.

Stocks of 10 mg/ml of tropomyosin (Tm) and troponin (Tn) (both: Lyophilised powder, rabbit muscle, Sigma Chemical Co., St. Louis, MO) were made in 10 mM TrisHCl pH 7.5, 1 mM DTT, 0.01% NaN₃, 5 mg/ml each of L-1-tosylamide 2-phenylethyl chloromethyl ketone (TPCK) and Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK), 0.3 mM phenylmethylsulphonyl fluoride (PMSF) [modified from Homsher et al., 1996] and stored at -25 °C for up to 3 months.
Effect of doxorubicin on actin-myosin interaction

Protein concentrations were measured by absorption spectroscopy or by protein assay (Bio-Rad). The molecular mass and extinction coefficient used to assay actin concentration were respectively 42 kDa and 0.62 \( (\text{mg/ml})^{-1} \text{cm}^{-1} \) at 290 nm.

**Solutions** [Kron et al., 1991]

Motility assays were carried out either with labelled actin filaments or with labelled thin filaments diluted in AB-buffer (pH 7.4, ionic strength 50 mM and pCa 10, 10 mM MOPS; 1 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetate (EGTA), 2 mM \( \text{Mg}^{2+} \), 2 mM Creatine Phosphate (Boehringer Mannheim, Germany) and 10 mM DTT. The ionic strength was adjusted by addition of KCl. Before use 35 U/ml Creatine Kinase (Boehringer Mannheim, Germany) was added to AB-buffer.

In the *in vitro* motility assay, movement of F-actin was induced by AB-buffer containing 2 mM \( \text{MgATP} \) and variable calcium concentrations. Appropriate amounts of \( \text{CaCl}_2 \) were added to obtain solutions with values of pCa between 10 and 4, while maintaining ionic strength. Scavenger solutions were used to minimise photooxidation and photobleaching. Therefore 3 mg/ml glucose, 10-25 U/ml glucose oxidase (Sigma Chemical Co., St. Louis, MO) and 200-500 U/ml catalase (Sigma Chemical Co., St. Louis, MO) were added to AB-buffer containing labelled actin or thin filaments (see Slide preparation for use of this buffer) and/or ATP.

Doxorubicin (Adriblastina® RTU, Doxorubicinehydrochloride, Farmitalia Carlo Erba, Belgium), typically 1 \( \mu \text{M} \), was added to the ATP containing AB-buffer just before infusion into the flow cell.

A computer program based on data of Fabiato and Fabiato [1979] was used to calculate the composition of the solutions to maintain ionic strength.

**Slide preparation**

Motility assays were carried out on glass. Therefor, flow cells were constructed from a no. 1 cover slip (24 x 40 mm), two spacers (each 3 x 24 mm) and a top cover slip (24 x 24 mm) attached to each other with grease. Cover slips were coated with a thin layer of freshly diluted nitrocellulose (1%) and used the same day [Kron et al., 1991]. Total chamber volume was about 100 \( \mu \text{l} \). All solutions were infused by pipette while holding the flow cell slightly tilted. Capillary forces facilitated the filling procedure. First, the flow cell was filled with 100-200 \( \mu \text{g/ml} \) heavy meromyosin (HMM) and incubated for 1
minute, followed by AB (buffer described in Solutions) with 10 mM bovine serum albumin (BSA) to remove unbound HMM and to block non-specific protein binding. After exposing the chamber to AB containing 0.02 μM unlabelled F-actin for 1 minute, the chamber was washed with AB containing 2 mM MgATP, to unblock active actin-myosin interactions and remove access of unlabelled F-actin, followed by two times AB with 10 mM BSA. This procedure uses unlabelled F-actin to block ATP-insensitive heads on HMM, which were either not removed by centrifugation (see Myosin and Heavy Meromyosin Preparation) or were formed when HMM bound to the nitrocellulose surface [Kron et al., 1991; Sellers et al., 1993]. Actin or thin filaments (4 nM) in AB was added and after 1 minute washed with either AB containing the scavenger solutions alone or, in case of thin filaments, with AB containing scavenger solutions plus troponin and tropomyosin (80 and 100 nM respectively). Finally, the slide was transferred to the microscope stage. Adding AB containing ATP (and in case of thin filaments also calcium, troponin and tropomyosin) starts the movement of the actin (or thin) filaments. All measurements are done at room temperature.

Data acquisition and analysis

Labelled actin filaments were observed through an inverted microscope (Nikon Diaphot 300) equipped with a Hg100W lamp and a Nikon fluorescence filter set (Omega Optical XF37; λexc = 546 nm, beam splitter 560 nm, λem = 570 nm). Actin filaments were imaged with an intensified CCD camera (Photonic Science Limited Darkstar). The standard PAL video signal from the camera, together with time/date/title signal (Blaupunkt), was recorded on an S-VHS video recorder (VCR model GV 470 S VPT Grundig) for off-line analysis.

To digitise the recorded images the video signal from the video recorder was fed into the S-VHS input of the standard video device (VINO) of a Silicon Graphics Indy workstation.

Actin filament speed was calculated using a home written program (ActinFil; written by JGZ) as described in Chapter 3. Only uniform sliding filaments were used for speed analysis. This excludes waving and buckling filaments, in which parts of these filaments are not attached to the myosin-surface.

For all conditions measured at least five areas from at least 3 slides were analysed. Results are presented as mean ± SE. For statistical analysis of speed of motile and non-
motile filaments, and percentage motile filaments all data were used. Statistical significance was determined by ANOVA followed by pairwise comparisons with the Tukey HSD test with a significance level of p<0.05.

RESULTS

As already described in Chapter 4 the movement of pure actin filaments appeared to be independent of calcium concentration in the range of pCa 10 to 4. In figure 5.1 speed of motile and non-motile actin filaments (A) and percentage of motile actin filaments (B) is shown at pCa 10 only, for comparison with the experiments with thin filaments. To maintain proper calcium regulation during the experiments troponin and tropomyosin were added to the buffer solutions, similar to the experiments of Homsher et al. [1996] and Gordon et al. [1997] for thin filaments reconstituted from skeletal muscle actin filaments with respectively cardiac and skeletal muscle troponin and tropomyosin.

For these untreated1 reconstructed thin filaments, all experiments started at pCa 4 and ended at pCa 10; verification afterward with pCa 4 led to a full recovery of speed and percentage of motile filaments. This indicates that the proteins were not damaged during the experiment. In experiments with doxorubicin-treated thin filaments, on verification with pCa 4 afterward, percentage of motile filaments and speed did not recover to the value for untreated thin filaments.

The path reconstruction method, described briefly in ‘Materials and Methods’ of this Chapter and in detail in Chapter 3, is used for speed analysis. Speed histograms obtained from the measurements show a clear minimum at 1 μm/s for all measured calcium concentrations (not shown here). This allows us to separate motile and non-motile filaments.

Figure 5.1A shows speed of motile and non-motile thin filaments at different calcium concentrations with and without doxorubicin, sliding over skeletal muscle HMM. At pCa 10 movement of thin filaments ceased. Also shown is speed of motile and non-motile actin filaments at pCa 10 with and without doxorubicin. There were no reliable measurements of speed and percentage of motile thin filaments with doxorubicin at pCa 9.

1 Untreated (or treated) is related to the use of a calcium antagonist only.
Speed of untreated motile thin filaments shows a decrease with decreasing calcium concentration (see also Chapter 4 for detailed description). Speed of motile thin filaments exposed to doxorubicin show the same tendency, but at a significantly higher speed level. Speed of non-motile thin filaments is significantly increased by doxorubicin as well. Doxorubicin has no effect on speed of motile and non-motile actin filaments.

**Figure 5.1** Speed and percentage of motile and non-motile rabbit skeletal actin and thin filaments with (1µM) and without doxorubicin, moving over rabbit skeletal HMM molecules, dependent on pCa.

A. Sliding speed (µm/s) of motile (◊) and non-motile (△) thin filaments at various pCa. The numbers of motile filaments analysed at pCa 4, 5, 6, 7, 9 and 10 were 187, 29, 35, 34, 9 and 0 respectively. The numbers of non-motile filaments analysed at pCa 4, 5, 6, 7 and 9 were 249, 103, 111, 192, 183, and 49 respectively. Sliding speed (µm/s) of motile (◊) and non-motile (△) thin filaments exposed to doxorubicin at various pCa. The numbers of motile...
Effect of doxorubicin on actin-myosin interaction

filaments analysed at pCa 4, 5, 6, 7, and 8 and 10 were, 44, 65, 56, 38, 29 and 0 respectively. The numbers of non-motile filaments analysed at pCa 4, 5, 6, 7, 8 and 10 were, 51, 38, 74, 34, 79 and 56 respectively. Sliding speed (μm/s) of motile (○) and non-motile (■) actin filaments is shown at pCa 10 using a line parallel to the pCa-axis to illustrate that this speed is not dependent on calcium. The numbers of motile and non-motile filaments analysed were 41 and 18 respectively. Sliding speed (μm/s) of motile (○) and non-motile (■) actin filaments exposed to doxorubicin is shown at pCa 10. The numbers of motile and non-motile filaments analysed were 62 and 74 respectively.

B. Percentage (%) of untreated (●) and doxorubicin-treated (○) motile thin filaments at various pCa. The numbers of untreated thin filaments analysed for pCa 4, 5, 6, 7, and 9, were 436, 132, 146, 226, 192 and 49 respectively (sum of the numbers of non-motile and motile filaments as mentioned in A). The numbers of doxorubicin-treated thin filaments analysed at pCa 4, 5, 6, 7, 8, and 10 were 95, 103, 130, 72, 108 and 56 respectively. Also shown is percentage motile actin filaments with (○) or without (●) doxorubicin. Numbers of untreated and doxorubicin-treated actin filaments were 59 and 136 respectively.

Figure 5.1B shows the percentage of untreated and doxorubicin-treated motile thin filaments at different calcium concentrations, and of untreated and doxorubicin-treated motile actin filaments. The percentage of doxorubicin-treated motile thin filaments decreases with decreasing calcium concentration, but remains at a significantly higher level compared with percentage motile filaments without doxorubicin. Percentage of doxorubicin-treated motile actin filaments is significantly decreased when compared to untreated motile actin filaments.

In figure 5.2A it is shown that doxorubicin has no effect on speed of actin filaments moving over rabbit skeletal muscle HMM, whereas figure 5.2B shows a significant increase in speed of actin filaments moving over rat heart muscle HMM.

Figure 5.2C and D show that addition of doxorubicin respectively significantly decreases percentage of motile actin filaments moving over rabbit skeletal muscle HMM and significantly increases percentage of motile filaments moving over rat heart muscle HMM.

There is no effect of doxorubicin on the myosin ATPase activity (for both skeletal and heart muscle myosin) measured in solution in presence and in absence of actin or thin filaments at pCa4 and 10.

At clinical use concentrations of 10 μM can be found in cells. Unfortunately, in the motility assays concentrations of doxorubicin above the 1 μM can not be used, since the intensity of autofluorescence exceeds the intensity of actin filament fluorescence.
During the experiments no remarkable breaking into segments of actin filaments were seen after addition of doxorubicin. Also G-actin could be polymerised in presence of doxorubicin.

![Graph](image)

**Figure 5.2** Speed and percentage of motile and non-motile rabbit skeletal actin filaments with (1 µM) and without doxorubicin, moving over rabbit skeletal or rat cardiac HMM molecules.

A. Speed (µm/s) of motile actin filaments moving over skeletal muscle HMM molecules with (in the darker shade of grey) and without doxorubicin. The numbers of motile filaments analysed were 144 and 31 respectively. B. Speed (µm/s) of motile actin filaments moving over cardiac muscle HMM molecules with (in the darker shade of grey) and without doxorubicin. The numbers of motile filaments analysed were 113 and 430 respectively. C. Percentage motile filaments (%) of actin filaments moving over skeletal muscle HMM molecules with (in darker shade of grey) and without doxorubicin. The numbers of filaments analysed were 261 and 76 respectively. D. Percentage motile filaments (%) of actin filaments moving over cardiac muscle HMM molecules with (in darker shade of grey) and without doxorubicin. The numbers of filaments analysed were 173 and 449 respectively.

**CONCLUSION AND DISCUSSION**

In this chapter the in vitro motility assay is used to study the direct effect of doxorubicin on the actin-myosin interaction. To ensure saturation of the actin filament with the regulatory proteins it is necessary to have the thin filament-proteins present in the motility buffer at a concentration greater than that of the actin. In control experiments speed of motile thin filaments increases with increasing calcium concentration. At pCa 5
Effect of doxorubicin on actin-myosin interaction

speed and percentage of thin filaments is maximal. At pCa 10 all movement ceased. This shows that all actin molecules in the thin filaments were subject to the regulating influence of the troponin-tropomyosin complexes. This graded behaviour of thin filaments is described in detail in Chapter 4 (Conclusion and Discussion). The concept of filament motility sketched in that chapter makes it possible to explain the results of measurement of percentage motile filaments and speed of untreated and doxorubicin-treated filaments.

On actin filaments sliding over rabbit skeletal HMM, doxorubicin has no effect on speed of motile and non-motile filaments, but it decreases the percentage of motile filaments. This suggests a direct effect of doxorubicin on the actin-myosin interaction, possibly directly on myosin or actin. One possibility is that doxorubicin occupies the troponin and tropomyosin binding sites of actin, thereby prohibiting movement, in a similar way as the troponin-tropomyosin complex without calcium. The actin- and ATP binding sites of myosin are less likely because no remarkable change in actin filament density is observed as well as any difference in actin filament speed.

A significant higher speed could be found for non-motile thin filaments after exposure to doxorubicin. Speed of non-motile thin filaments with and without doxorubicin was also dependent on calcium. This suggests a decrease in weak binding affinity by doxorubicin and by calcium, causing an increase in Brownian motion contributing to the speed of non-motile filaments.

On thin filaments sliding over rabbit skeletal HMM, doxorubicin treatment leads to a higher percentage motile thin filaments and a higher speed at all calcium concentrations. Speed and percentage of motile untreated and treated thin filaments are dependent on pCa and the slopes of the curves are not significantly different. No change in ATPase activity is measured but according to Gordon et al. [1997] an increase in speed does not necessarily have to be accompanied by an increase in ATPase activity. Doxorubicin can reduce friction from the troponin-tropomyosin complex or enhance the calcium release and binding rate. We can imagine this as a facilitated accessibility of actin filaments to myosin, without changing the calcium dependence, or as a facilitated binding of actin to myosin through an effect on myosin. In both cases, doxorubicin would be recruiting more active cross-bridges. Also an enhanced cooperativity between the troponin-tropomyosin complex could explain an increase in active cross-bridges and thus motility (speed and percentage of motile thin filaments).
The positive effect of doxorubicin on the percentage of motile thin filaments is opposite to the negative effect of doxorubicin on the percentage of motile actin filaments. This suggests that the effect on actin filaments is counteracted by the effect on thin filaments, or that the effect of doxorubicin on actin filaments is prohibited by the troponin-tropomyosin complex, i.e. the binding sites for the troponin-tropomyosin complex are already occupied.

As described in Chapter 4 an explanation for the decreased percentage of motile thin filaments and maximum speed compared to motile actin filaments is to assume an extra friction caused by the troponin-tropomyosin complex or an influence of the calcium release and binding rate. Doxorubicin, which shows a comparable effect as verapamil in Chapter 4, has the ability to increase percentage motile filaments and speed to a level comparable to speed and percentage of actin filaments. This can be explained by a release of friction from troponin-tropomyosin complexes or an enhanced cooperativity between the troponin-tropomyosin complexes.

This direct effect of doxorubicin on thin filaments, moving over rabbit skeletal HMM, is in agreement with deBeer et al. [1992] who showed a direct effect of doxorubicin on skeletal muscle fibres. They observed a small right-sided shift of the calcium sensitivity curve. They suggested a direct interaction of doxorubicin with the actin-myosin structure possibly by an effect on the ATPase activity or a disrupture of the actin-myosin structure. Also Doroshow et al. [1985] showed ultrastructural damage to heart and skeletal muscle in mice after 4 days of administration. They showed that the degree of myocyte damage was dependent upon the doxorubicin concentration in the different tissues used and that there was much less uptake of doxorubicin in skeletal than in heart muscle. These results indicate that if acute supply of doxorubicin causes a disrupture of the actin-myosin structure, like proposed by deBeer and Doroshow, it will probably concern the disrupture of troponin and tropomyosin around the actin filaments in both skeletal and heart muscle preparations. A possibility would be that this disrupture can be accompanied or starts with a change in position of tropomyosin molecules, making the actin filament more accessible for myosin. This also explains the increase in percentage motile filaments we found, even at low calcium concentration.

In our experiments a remarkable difference in effect of doxorubicin on motile actin filaments moving over heart and skeletal muscle HMM was found (as described in Results and shown in figure 5.2). Doxorubicin increases speed and percentage of actin
filaments moving over heart muscle HMM and decreases percentage of actin filaments moving over skeletal muscle HMM. This indicates that besides the higher uptake of doxorubicin in heart muscle [Doroshow, 1985], there is possibly also a higher sensitivity to doxorubicin of heart muscle myosin than of skeletal muscle myosin. However, we can not exclude that this effect is only related to the different species used.

Because the experiments with doxorubicin were not reversible, it is likely that doxorubicin causes structural changes in myosin heads or actin that influence results. This damage may be clinically relevant but is hard to interpret. Although the mechanism of cardiac toxicity is not fully understood, free radical-mediated cell injury may play an important role in its pathogenesis [Doroshow, 1983]. The generation of oxygen free radicals is facilitated by the formation of doxorubicin-iron complexes. In our system however, there are hardly any iron ions present and oxygen radicals are removed by the scavenger system present. Therefore, damage to actin or myosin in the *in vitro* motility assay can only be a result of doxorubicin or a metabolite thereof.

According to Bottone *et al.* [1998] doxorubicin shows a biphasic action of doxorubicin on rat heart muscle fibres. After acute exposure an increase in tension is measured whereas after chronic exposure an impairment of tension is measured. The increase in speed and percentage of motile actin filaments after acute exposure to doxorubicin, seen in our experiments with both rat heart muscle HMM and rabbit skeletal muscle HMM, seem to mimic the first phase described by Bottone. The irreversibility of our experiment leads to an impairment of speed and percentage motile filaments, which is comparable with the second phase. This biphasic action can be explained by a positive inotropic effect in the first phase leading to disruption of the contractile machinery in a later stage (second phase).

During our experiments no remarkable breaking into segments of actin filaments were seen after addition of doxorubicin. Also G-actin could be polymerised in presence of doxorubicin. Although Dalledonne *et al.* [1992, 1993], Colombo *et al.* [1988] and Colombo and Milzani [1988] showed that doxorubicin decreases size of actin polymers and actin polymerisation, no effect was found here. According to Colombo *et al.* [1990] however these processes are highly dependent on metal ion composition. In presence of Mg²⁺ (and KCl) and at low doxorubicin concentration, which are exactly the conditions in the *in vitro* motility assay, actin polymerisation and actin filament size is hardly affected. Also the fluorescent label of actin filaments, rhodamine phalloidin, used in the *in vitro*
motility assay and also present in the polymerisation experiment, stabilises the actin filaments.

No changes in ATPase activity from exposure to doxorubicin have been measured. This implies that although percentage motile filaments and speed were higher the energy expenditure did not change.

In conclusion, the positive inotropic effect found from acute exposure to doxorubicin on rat heart muscle and rabbit skeletal muscle as reported by Bottone et al. [1997] and deBeer et al. [1992] is also found in our experiments. The positive inotropic effect on actin filaments moving over rat heart muscle HMM can be related to a direct effect on the actin-myosin interaction. The positive inotropic effect of doxorubicin on thin filaments moving over rabbit skeletal muscle HMM can be related to a released friction of the troponin-tropomyosin complex or an increase in cooperativity between these troponin-tropomyosin complexes. However, we found a negative inotropic effect from acute exposure to doxorubicin on actin filaments moving over rabbit skeletal muscle HMM. This could also be related to a direct effect on the actin-myosin interaction, but opposite to the effect seen on actin filaments moving over rat heart muscle HMM.

Future experiments determining the binding site of doxorubicin in experiments interchanging tropomyosin, and troponin, combined with measurements in which actin filaments are put under load might lead to answers concerning acute force increase, ATPase activities and effects of tropomyosin, troponin and light chains.
Effect of doxorubicin on actin-myosin interaction

References


