Interaction of Flavonoid Compounds with Contractile Proteins of Skeletal Muscle

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Abstract. Flavonoids (quercetin, rutin) influence ATPase activity and actomyosin superprecipitation. Low concentrations (below 20 μmol/l) of flavonoids were found to cause conformational changes in the myosin structure accompanied by an increase in ATPase activity. At higher concentrations an inhibitory action of flavonoids on both ATPase activity and actomyosin superprecipitation occurred. Conformational changes are likely to be due to flavonoids binding to regulatory site near the active centre of the myosin head. The effect of quercetin was stronger than that of rutin.

Key words: Flavonoids — Quercetin — Rutin — Myosin — Actomyosin

Introduction

Flavonoid compounds are well known to effect the functional behaviour of contractile systems. These drugs decrease smooth muscle tone, and decrease frequency and increase amplitude of heart beats (McClure 1975). Considerable attention is paid nowadays to studies of molecular mechanisms of the flavonoids actions on cellular processes. One flavonoid, quercetin, has been found to suppress both mitochondrial and chloroplast ATPases causing inhibitions of bioenergetic processes (Lang and Racker 1974; Fewtrell and Gömperts 1977). Both, ATPase activity and Ca^{2+} uptake of the sarcoplasmic reticulum (SR) are inhibited (Shoshan and McLennan 1981). Quercetin (100 μmol/l) caused an 85 % decrease in Ca^{2+} uptake by SR; this along with a facilitation of Ca^{2+} release, caused threefold increase in the rate of tension development of skinned muscle fibres (Shoshan et al. 1980.; Watras et al. 1983).

Experimental data have clearly shown that quercetin has a significant inhibitory effect on ATPase of various biological objects. At the same time no data concerning the action of quercetin and other flavonoids on muscular myosin ATPase have been available.

In the present study effects of quercetin and rutin on myosin ATPase activity and actomyosin superprecipitation as well as on conformational states of these contractile proteins were studied. Flavonoids at low concentrations (below 20μmol/l) were found to cause some conformational changes in myosin accompanied by activation of its ATPase. Inhibition rather than activation of both myosin ATPase and actomyosin superprecipitation were seen at higher concentrations of flavonoids (above 20μmol/l).

Materials and Methods

Chemicals. Quercetin and rutin were from „Reachim" (USSR), 1-anilino-8-naphtalene sulfonate (ANS) was from „Sigma" (USA). All other chemicals were of reagent grade.

Protein preparation. Rabbit skeletal muscle myosin was preparad by the method of Perry (1955), as modified by Tartakovsky (1978). In brief this technique has following steps: 1. Short-time extraction of muscle proteins with high ionic strength solution. This solution contained pyrophosphate and magnesium ions to avoid an increase of the actomyosin fraction in the extract. 2. Myosin precipitation with decreased ionic strength to remove sarcoplasmic proteins ($i = 0.04$) 3. Removal of actomyosin remainder by centrifugation at 100,000 × g, for 1h, $I = 0.3$. 4. Myosin precipitation at low ionic strength ($I = 0.04$). 5. Clarification of the myosin solution by high-speed centrifugation (105,000 × g, for 1h, $I = 0.5$).

Preparation of actomyosin according to Bárány et al. (1966): Extracts were prepared from homogenized muscle with high ionic strength salt solution ($I = 0.5$) during 24 h. Then, the extracts were fourfold precipitated and washed with solutions with changing ionic strength (from 0.5 to 0.05) to remove water-soluble contaminants and regulatory proteins. Finally, actomyosin was dissolved in 0.5 mol/l KCl, 5 mmol/l Tris-HCl, pH 7.5 and centrifuged at 40,000 × g for 1 h.

Analytical methods. The protein concentration was estimated by Biuret reaction. Protein preparations were checked for purity and homogeneity by PAAG electrophoresis in the presence of sodium dodecyl sulfate (SDS) (Weber and Osborn 1969). The nucleic acid contamination was estimated by determining the ratio of light absorption at 280 and 260 nm.

ATPase activity assay. In studying effects of flavonoids on contractile proteins, the ATPase activity was determined by measuring the increase in medium acidity as a result of proton release from orthophosphoric acid formed at ATP hydrolysis. The kinetics of proton release into the medium must correspond to that of ATPase reaction in its initial stage. This method was proposed by Green

and Mommaerts (1953) to study muscle adenosine-triphosphatases, and later it was successfully applied and modified by other scientists (Zacepina and Schnol 1965; Eisenberg and Moos 1967; Stein et al. 1979). The main features of the method are its high sensitivity, rapidity and reproducibility of results.

We worked with a pH-meter type LPU-01 (USSR) and records were made using an XY-plotter. Thermostable cuvettes (10 ml) with magnetic stirrers were used. The reaction mixture contained 0.5 mol/l KCl; 25 mmol/l Tris-HCl; pH 7.5; 5 mmol/l $CaCl_2$; 50 μmol/l ATP; and 0.5 mg/ml myosin or actomyosin. The reaction temperature was 37°C. To estimate inorganic phosphate on the basis of proton release, the equivalence ratio as reported by Green and Mommaerts (1953) was used.

Actomyosin superprecipitation was followed by changes in absorption at 660 nm.

Quercetin, rutin and ANS were dissolved in 96 % alcohol and added into cuvettes containing reaction mixture so that the final alcohol concentration in the probes did not exceed 3 % to avoid protein denaturation. Myosin and actomyosin ATPase activity and actomyosin superprecipitation were not influenced at the alcohol concentrations used.

Fluorescence measurements. Fluorescence spectra were recorded using and Hitachi MPF-3 fluorescent spectrophotometer (Japan). A sensitive biwave fluorescent method (Turoverov and Schelchkov 1970; Zyma et al. 1977) was used to study conformational changes in myosin molecules.

Protein ultraviolet fluorescence was excited at 280 nm and ANS fluorescence at 365 nm.

Results were expressed as mean $\pm$ S. D. Statistical significance was assessed by Student's test, and probabilities of less than 5 % were considered significant.

Results

Qualitative SDS electrophoretic analysis of myosin and actomyosin preparations did not reveal any additive zones corresponding to contaminating proteins. Myosin electrophoreograms showed a single band corresponding to myosin heavy chains (200 kDa) and three bands (17, 20 and 25 kDa) corresponding to light chains. Actomyosin electrophoreograms also contained a band of actin (43 kDa). The preparations contained no troponin or tropomyosin. The optical density ratio D_{280} / D_{260} for myosin and actomyosin preparations was 1.6—1.8, suggesting that there was not nucleic acid contamination.

The nativity of the protein preparations was suggested by sufficient ATPase activities 0.53 $\pm$ 0.03 μmol $P_i \cdot mg^{-1}min^{-1}$ for myosin ($n = 13$) and 0.64 $\pm$ 0.03

μmol P_i.mg^{-1} . min^{-1} for actomyosin (n = 12). The reaction mixture contained 0.5 mol/l KCl; 20 mmol/l histidine buffer (pH 7.5); 5 mmol/l $CaCl_2$; 1 mmol/l ATP; 0.5 mg/ml protein in an assay probe, $t°$ = 37°C. In this case, inorganic phosphorus was estimated by the methods of Fiske and Subbarow (1925).

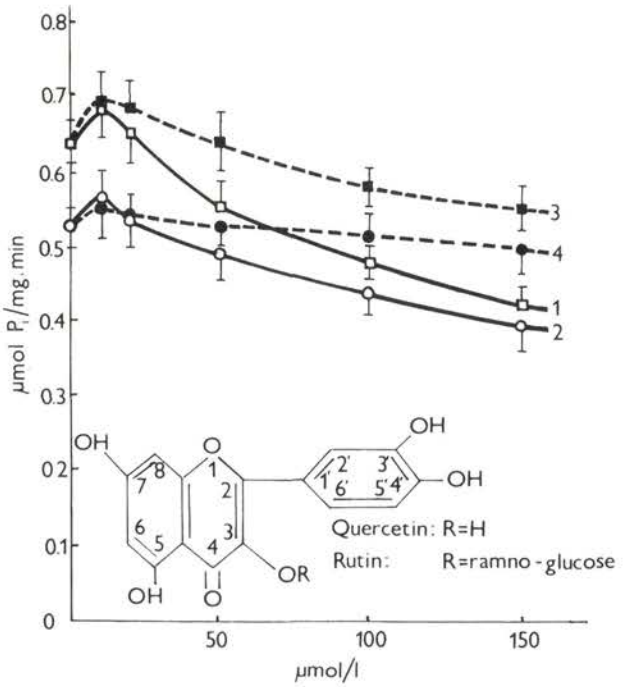

Fig. 1. Dependence of ATPase activity (1,3-actomyosin, 2,4-myosin) on flavonoids concentration: 1,2-quercetin; 3,4-rutin. The ATPase assay conditions were as follows: 0.5 mol/l KCl, 25 mmol/l Tris-HCl, pH 7.5; 5 mmol/l $CaCl_2$, 50 μmol/l ATP, 0.5 mg/ml myosin or actomyosin. Temperature: 37°C. Points are means $\pm$ S. D. (n = 12).

In view of the above parameters our myosin and actomyosin preparations could be considered as pure and suitable for further investigations.

Fig. 1 (curves 1 and 3) shows the dependence of skeletal muscle actomyosin ATPase activity on flavonoid concentration. Low concentrations of flavonoids (below 20 μmol/l) had activatory action on actomyosin ATPase. Higher concentrations of flavonoids inhibited actomyosin ATPase activity. In the presence of 150 μmol/l of quercetin the actomyosin ATPase activity decreased 1.52 times as compared to control values. Rutin was somewhat weaker the inhibition coefficient being only 1.17.

Similarly as actomyosin ATPase, the myosin ATPase activity was also activated by low concentrations of quercetin and rutin (Fig. 1, curves 2 and 4).

Although higher concentrations of quercetin were also depressive, the inhibitory activity was weaker on myosin ATPase than on the actomyosin complex. Quercetin (150 μmol/l) caused a drop with a coefficient of 1.36 only. The effect of rutin on myosin ATPase was still weaker. While low rutin concentration activated myosin ATPase, higher concentrations of the drug inhibited the activity.

Kinetic Lineweaver-Burk plots provided dissociation constants (Km) of the enzyme-substrate complex both in absence and presence of quercetin in solution (Fig. 2). In either case the constant was 43 μmol/l. This suggests a non-competitive mode of interaction between quercetin and either actomyosin or myosin ATPase. A value of 96 μmol/l was found for non-competitive inhibition constant K_i for actomyosin, and 320 μmol/l for myosin.

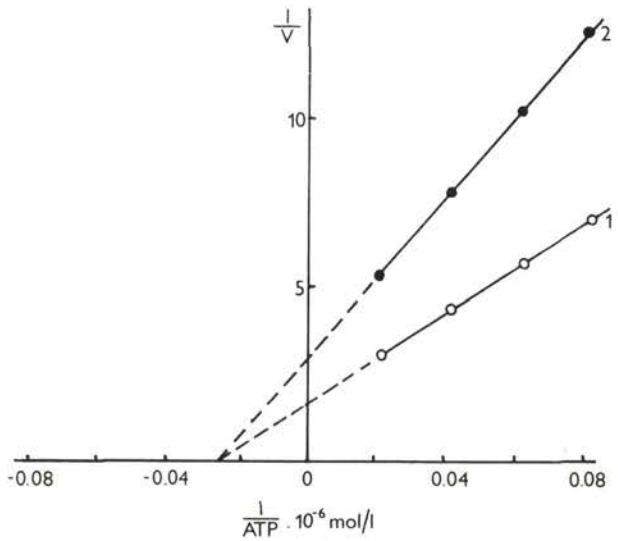

Fig. 2. Lineweaver-Burk plots of kinetic data of ATP hydrolysis by actomyosin in absence (1) and presence (2) of 100 μmol/l of quercetin. V-ATPase activity in arbitrary units.

Superprecipitation is one important characteristic of the actomyosin complex normally initiated at low ionic strength ($I = 0.1$), and can be observed as a fast increase in light scattering of actomyosin solution upon ATP addition (Ebashi 1961). This superprecipitation is thought to imitate muscular contraction. The effects of various concentrations of flavonoids on the extent of actomyosin superprecipitation were studied (maximal change in optical density of actomyosin solution after the initiation of superprecipitation). Fig. 3 shows the dependence of the actomyosin superprecipitation rate on quercetin and on rutin concentration. Low quercetin concentrations (up to 10 μmol/l) had an

only slight suppressive influence on actomyosin superprecipitation. However, concentrations ranging between 10—50 μmol/l strongly inhibited actomyosin superprecipitation. A more than 4-fold decrease of superprecipitation rate was observed in the presence of 50 μmol/l quercetine. Further increasing drug concentration was not followed by further enhancement of the inhibition. Rutin at low concentrations (up to 10 μmol/l) was found to activate both actomyosin superprecipitation rate and ATPase activity. A slight inhibition of actomyosin superprecipitation was observed at higher rutin concentrations (above 10 μmol/l). A 20 % decrease of superprecipitation rate was observed with 100 μmol/l of rutin.

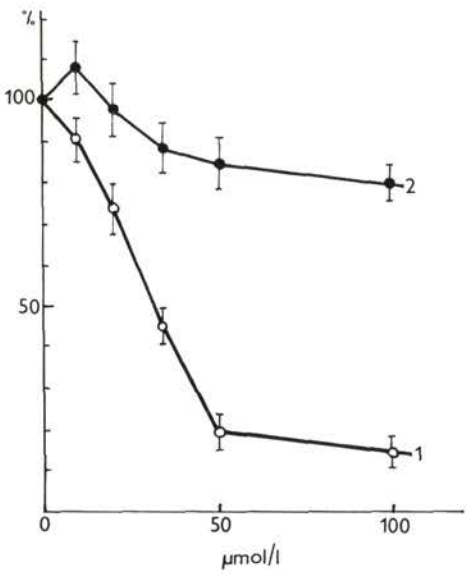

Fig. 3. Dependence of actomyosin superprecipitation rate on quercetin (1) and rutin (2) concentration. The superprecipitation rate in absence of flavonoids was taken as 100 per cent. Points are means ± S. D. ($n = 9$).

The results obtained suggest that flavonoids are likely to bind to some sites on myosin ATPase and those for actin binding.

Ultraviolet (UV) difference absorption spectra with maxima at 238 and 375 nm seem to prove that flavonoids bind to some sites on the myosin molecule.

Although flavonoids show strong light absorption (for quercetin $E_{385} = 16.1.10^3 \,\mathrm{mol}.\mathrm{l}^{-1}\mathrm{cm}^{-1}$) these drugs lack fluorescence. A fluorescent dye ANS was used to study the effects of quercetin and rutin on the conformational states of both the myosin and the actomyosin complex.

Ultraviolet fluorescence of tryptophane residues with $\lambda_{max} = 333$ nm is normally seen upon excitation of actomyosin and myosin at $\lambda = 280$ nm (Fig. 4, curve 1). With ANS added an additional fluorescent spectrum of ANS with $\lambda_{max} = 473$ nm is always seen (Fig. 4, curve 2). In the presence of quercetin in

actomyosin solution with ANS added a decrease in quantum yield and a blue shift of the actomyosin intrinsic fluorescent spectrum was observed while ANS fluorescent spectrum showed a red shift (Fig. 4, curves 3 and 4). Basically, the same results were found for rutin although its effect was much weaker. It is evident especially for ANS spectrum which showed only 4 nm red shift for rutin and 12 nm shift for quercetin (both drugs at 50μmol/l). The spectral changes observed are likely associated with some effects of the drugs on the actomyosin structure.

Structural analysis of actomyosin or myosin based on fluorescence intensity measurements is impossible due to screening of excitation light by flavonoids which is especially significant at higher concentrations of these drugs. The problem can be easily overcome by employing the highly sensitive two-wave method of fluorescence spectra recording.

Flavonoid-induced changes in the so-called two-wave parameter $B = I_{320} / I_{348}$, where I is the intensity of UV fluorescence of actomyosin at 320 and 348 nm respectively are shown in Fig. 5. An increase in parameter B following increasing quercetin or rutin concentrations is evident. This increase, observed for both

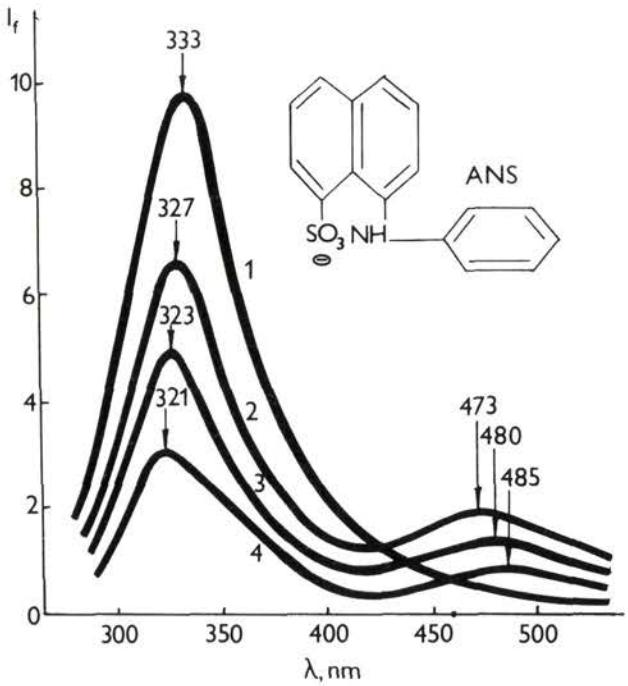

Fig. 4. Fluorescence spectra: 1-actomyosin, 2-actomyosin + ANS (20μmol/l); 3-actomyosin + ANS (20μmol/l) + quercetin (10 μmol/l); 4-actomyosin + ANS (20 μmol/l) + quercetin (50μmol/l). Actomyosin concentration was 0.5 mg/ml. I_f — fluorescence intensity in arbitrary units.

actomyosin and myosin in similar experimental conditions is due to conformational changes resulting in transition of tryptophanyl in more hydrophobic environment. The most significant changes in parameter B were observed with low concentrations of quercetin and or rutin (below 20 μmol/l). These conformational changes in myosin and actomyosin induced by low concentrations of flavonoids result in ATPase activation (Fig. 1). The increase in parameter B was slower with flavonoid concentrations above 20 μmol/l.

The behaviour of ANS reflects structural changes occurring exclusively in defined site of the macromolecule studied. It has been shown previously (Cheung and Morales 1969; Bogach et al. 1976) that 1 ANS molecule binds 1 head of a myosin molecule. This implies that ANS can be considered a specific dye to check local conformational changes in myosin heads. The so-called two-wave parameter $A = I_{440}/I_{513}$ (for ANS fluorescence) normally decreases when ANS molecules are exposed to more polar environment, and inversely (in alcohol-water solution, hydrophobic loci of proteins) (Bogach et al. 1978).

Fig. 5 (curve 3) shows a decrease of parameter A following an increase in quercetin concentration suggesting that ANS transition in more polar microenvironment has been enhanced.

Rutin binding to these sites is somewhat weaker since little change in parameter A was seen (Fig. 5, curve 4).

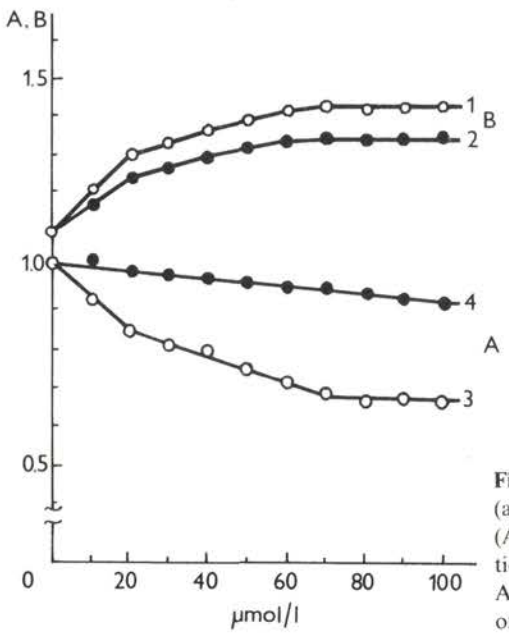

Fig. 5. Dependence of two-wave parameter B (actomyosin fluorescence) and parameter A (ANS fluorescence) on flavonoids concentration; 1 and 3 — quercetin, 2 and 4 — rutin. Actomyosin concentration was 0.5 mg/ml, that of ANS 20 μmol/l.

Discussion

The results obtained along with the similarity of the structures of quercetin and ANS suggest that flavonoids and ANS compete for the same binding site in the myosin head. These findings agree with observations on the ATPase coupling factor 1 of chloroplast membranes where quercetin was also found to compete with ANS (Cantley and Hammes 1976). ANS binding also caused some small conformational changes; however, ATPase activity remained unaltered. At the same time flavonoids, particularly quercetin, binding induced conformational changes with significant effect on ATPase activity. Rutin was less effective due to steric limitations: there is a large radical of ramno-glucose in position 3 of rutin while quercetin has an OH group in this position. It is this group in positions 3, 5, 3′, 4′ which determines biological activity of flavonoids (Lang and Racker 1974; McClure 1975).

Flavonoids are likely to specifically affect the groups located in the active site of myosin. It is well known that a number of chemicals such as DNP, dioxane, urea, ethyleneglycol, phormamid, or bisulphonic reagents have biphasic effect (Poglazov and Levitsky 1987). These drugs interfere with SH_1 — and SH_2 — groups adjacent to the active site of myosin. The active site of myosin ATPase in sited in a polypeptide located at the C-end moiety of the heavy chain of the myosin head near S-I-S-2 hinge (Elzinga and Collin 1977). EPR analysis showed that SH_1 and SH_2 groups are sited at 1.2—1.4 nm distance from each other and withdraw from the active site at a distance of 3 nm (Burke and Reisler 1977; Shukla and Levy 1978). Mg^{2+}–ADP binding to the active site makes SH_1 —SH_2 groups to move closer to each other. Due to this Mg^{2+}– ADP is tapped in the active site causing myosin inactivation.

Some data indicate that flavonoids cause conformational changes in protein molecules due to interactions with SH- and aminogroups (Carpenedo et al. 1969; Cantley and Hammes 1976). Based on this we suggested that binding of flavonoids to ANS sensitive sites allosterically modifies SH_1 and SH_2 groups, resulting in a change of enzymatic activity. Our data support the idea of a biphasic action of flavonoids on myosin ATPase. We observed an initial increase in parameter B induced by low concentrations of flavonoids (below 20 μmol/l). Higher concetrations showed the decrease of rise. Low concentrations of flavonoids also activated myosin ATP-ase and higher concentrations of the drugs inhibited it. Basically the same results were obtatined with SR (Ca^{2+} + Mg^{2+}) — ATPase (Shoshan and McLennan 1981).

In addition to an ATPase site the myosin head also contains a binding site for actin (Eisenberg et al. 1980). The active site and the actin binding site are not identical and are located in different parts of the myosin head. As a rule, actin binding brings about significant conformational changes around SH-groups

which are essential for normal ATPase activity (Kameyama 1980): it is obvious that there is a close functional relationship between these two sites.

We found that flavonoids binding to myosin heads has a stronger inhibitory effect on actomyosin superprecipitation than on ATPase activity. We suggest that the superprecipitation site and the actin binding site are either identical or closely located. This implies that in the presence of actin flavonoids cause significant conformational changes in myosin which promote suppression of superprecipitation sites.

A kinetic analysis of ATP hydrolysis showed that quercetin affects myosin ATPase activity by a noncompetitive way. This means that flavonoids are closely bound to the active site and allosterically affect the myosin active site.

We conclude that ANS, quercetin and rutin bind to the regulatory sites of myosin heads the sites including a hydrophobic locus which contains several aromatic aminoacid residues including tryptophanyls. Close to the regulatory site there are some groups (SH-group in particular) the modification of which influence ATP hydrolysis and superprecipitation. ANS molecule has a large hydrophobic surface enabling its effective binding to hydrophobic regions resulting in an increase in ANS fluorescence. ANS binding induces some conformational changes not involving SH-groups and resulting in no changes in actomyosin ATPase activity and/or superprecipitation. Quercetin also binds to the same sites thus inducing significant conformational changes. As a rule allosteric modification of SH-groups occurs associated with significant changes in actomyosin ATPase activity and/or superprecipitation. Due to steric limitations rutin induces smaller structural changes in myosin heads.

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