Biochimica et Biophysica Acta, 730 (1983) 245–254
Elsevier Biomedical Press

BBA 71633

INHIBITION OF Ca^{2+}-TRANSPORT ATPase FROM SYNAPTOSOMAL VESICLES BY FLAVONOIDS

ARI BARZILAI and HANNAH RAHAMIMOFF

Department of Biochemistry, Hebrew University - Hadassah Medical School, Jerusalem, 91010 (Israel)

(Received November 29th, 1982)

Key words: Ca^{2+}-ATPase inhibition; Flavonoid; Quercetin; Synaptosome; (Rat brain)

The inhibitory action of the flavonoid quercetin has been examined on the calcium-transport ATPase of synaptosomal vesicles and compared to that of two other flavonoids, morin and rutin. We have found that while quercetin caused a 50% inhibition of calcium transport at a concentration of 15 μM, morin and rutin had similar effects at concentrations of about 200 μM. A similar order of potency was observed also for ATP hydrolysis, though at higher concentrations. Quercetin also strongly inhibited phosphorylation of membrane proteins by ATP in synaptosomal vesicles. Rutin and morin had an almost negligible effect on membrane protein phosphorylation. The order of inhibitory potency of the flavonoids on the Ca^{2+}-transport ATPase from synaptosomal vesicles: quercetin > morin > rutin, could be linked to their possible solubility in the membrane lipid phase since: (1) it paralleled their partitioning between a mixture of oil and water; (2) it paralleled their uptake from the reaction mixture by synaptosomal vesicles and phosphatidylcholine liposomes; (3) they had almost equal potency as inhibitors of the water soluble system of histone phosphorylation by protein kinase.

Introduction

The flavonoids are a group of substances described as inhibitors of ion-transport ATPases. Among the ATPases investigated were ($Na^+ + K^+$)-ATPase [1,2], mitochondrial H^+-ATPase [1,3], sarcoplasmic reticulum Ca^{2+}-ATPase [4,5], red blood cell Ca^{2+} pump [6] and many more.

It has been suggested that quercetin (3,3′,4′,5,7-pentahydroxy flavone) uncouples ($Na^+ + K^+$)-ATPase activity by inhibiting ATP hydrolysis without altering Rb^+ translocation [7,8].

We investigated the inhibitory effects of flavones on the Ca^{2+} transport system in synaptosomal vesicles. Synaptosomal vesicles are small membrane vesicles isolated from lysed synaptosomes by gradient centrifugation. They transport Ca^{2+} very efficiently in an ATP-dependent fashion [9]. A divalent cation-dependent ATPase has been found associated with the calcium transport process [10,11]. This ATP-dependent transport activity has been extensively purified and reconstituted into phospholipid membranes [12]. Since the mechanism of action of synaptosomal vesicle Ca^{2+}-transport ATPase is not known and since no specific inhibitor of Ca^{2+}-transport ATPase is available, we decided to use quercetin and other flavones as a tool to explore its possible mechanisms of action.

In addition, investigation of the mode of action of Ca^{2+} transport inhibitors is of general interest, since this provides us with an experimental tool to study the regulation of intracellular Ca^{2+} concentration which is of paramount importance in the neurotransmitter liberation process [13].

In this article we examine the effect of quercetin and other flavonoids on the ATP-dependent Ca^{2+}-transport system of synaptosomal vesicles

and ATP mediated phosphorylation of the ATPase itself and other membrane proteins. In addition, we examine the relation between lipid solubility of the flavonoids and their relative potency as inhibitors.

Materials and Methods

Preparation of membrane fractions. Preparation of synaptosomal vesicles was done by differential gradient centrifugation from lysed synaptosomes obtained from brains of 14-day-old rats as described by Rahamimoff and Abramovitz [9]. In each preparation, 30 rat brains were combined. All the experiments described here were performed several times with at least three different preparations of membranes. The purification and reconstitution of the synaptosomal vesicle Ca^{2+}-transport system has been described in detail [12].

Calcium transport. Calcium transport was measured by two techniques: native synaptosomal vesicles were incubated for given time and conditions (see legends of appropriate figures and tables). The reactions were stopped by a 20-fold dilution with a buffer system identical to the one used in the reaction mixture – without the Ca^{2+}, ATP or other additions, and filtered rapidly through 0.45 μm Schleicher and Schuell nitrocellulose filters. The filters were washed two more times with the same buffer and counted in a liquid scintillation counter after drying. Reconstituted membranes were passed through a Dowex X-50 mini-column at the end of the reaction to separate intravesicular from extravesicular calcium, as described by Gasko et al. [14].

In all transport measurements, zero-time controls were subtracted from the values obtained to correct for binding of Ca^{2+} to the membranes. These values did not exceed 10% of the Ca^{2+} transport.

ATP hydrolysis. Hydrolysis was measured by determining the amount of $^{32}P_i$ (inorganic phosphate) liberated from [γ-^{32}P]ATP following incubation with the appropriate membrane fraction as described [15]. The data obtained were corrected for the self-hydrolysis of ATP obtained by incubating identical reaction mixtures, except that membranes were omitted.

Phosphorylation of membranes. Phosphorylation was performed by incubating the membranes in buffered KCl solution with 2 μM [γ-^{32}P]ATP for 20 s. The amount of Mg^{2+} was 30 μM. The reaction was terminated by addition of sodium dodecyl sulphate to a final concentration of 2% and the mixture was loaded on a Sephadex G-50 mini-column to separate the phosphorylated membranes from unhydrolyzed [γ-^{32}P]ATP or liberated $^{32}P_i$. The membranes excluded from the column were either counted or used for analysis by polyacrylamide gel electrophoresis.

Gel electrophoresis was done in a Laemmli [16] slab gel system. Phosphorylation of histone was performed by using protein kinase (catalytic subunit) from bovine heart or adrenal medulla as described by Kuo and Greengard [17].

Inhibition by flavonoids. Quercetin, morin or rutin were added to the membranes always 20 min before the reaction was started. The membranes were preincubated with the flavonoids at 37°C, and the concentrations used are specified for each experiment.

Liposome washes. Washes were done by adding 450 μg phosphatidylcholine liposomes, prepared as described by [18], to synaptosomal vesicles (15 μg protein) previously incubated with or without quercetin and preloaded with Ca^{2+} in an ATP-dependent manner. The initial inhibition with quercetin was determined. At the end of 20 min incubation, the liposomes were separated from synaptosomal vesicles by centrifugation at 30000 $\times g$ for 15 minutes (liposomes prepared by ultrasonic treatment do not pellet under these conditions). Synaptosomal vesicles were reincubated with $^{45}CaCl_2$ and ATP. Ca^{2+} transport was measured again and compared to the initial data obtained.

Materials. Quercetin, morin and rutin were obtained from Sigma Chemical Company and purified by recrystallization from ethanol. $^{45}CaCl_2$ and [γ-^{32}P]ATP were obtained from New England Nuclear. Bovine heart protein kinase (cAMP-independent) was from Sigma, and protein kinase from adrenal medulla was the generous gift of Drs. D. Oxsenberg and U. Bachrach, Department of Molecular Biology, Hebrew University Medical School. All other reagents used were analytical grade reagents.

Results

Inhibition of the synaptosomal vesicle Ca^{2+}-transport system by flavonoids

Synaptosomal vesicles transport calcium very efficiently when ATP is added to their extravesicular medium. The effects of quercetin, morin and rutin on the ATP-dependent Ca^{2+} transport and associated ATP hydrolysis have been investigated. In Fig. 1 the effect of the above flavones on the synaptosomal vesicle Ca^{2+}-transport system is shown. Fig. 1A shows that all three flavones have inhibitory action on the ATP-dependent Ca^{2+} transport of synaptosomal vesicles. But while quercetin, at a concentration of 15 μM, caused a 50% inhibition of the calcium transport, over 200 μM morin or rutin were required for a similar effect. Higher concentrations of flavones were required to inhibit ATP hydrolysis. Fig. 1B shows that 50% inhibition of ATP hydrolysis was obtained with 75 μM quercetin, while up to 500 μM morin and rutin yielded only 25% inhibition of ATP hydrolysis. The flavonoids had no effect on the Ca^{2+} associated with the vesicles in the absence of ATP, which did not exceed 20% of the total transport obtained in the presence of ATP, in any of the experiments.

The ATP-dependent Ca^{2+} entry into the vesicles represents active transport. This can be shown by various ways: The Ca^{2+} taken up by the vesicles at steady state under the conditions described in Fig. 1 is 6 nmol/mg protein. Since the intravesicular volume of these vesicles is 3 μl/mg protein [9], the intravesicular calcium concentration is at least 2 mM (assuming that all the vesicles take up Ca) – or more. Since the extravesicular calcium con-

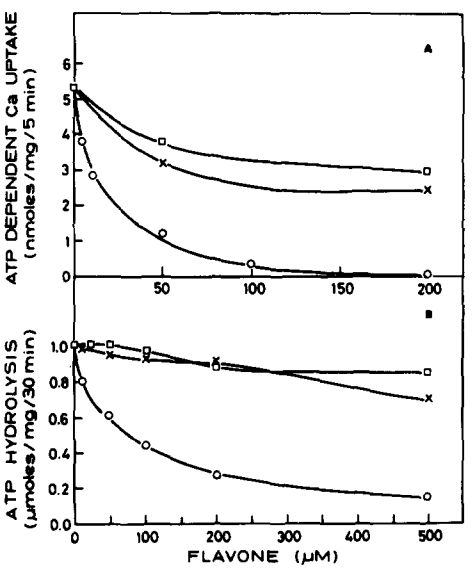

Fig. 1. A. The effect of flavonoids on ATP-dependent Ca^{2+} transport in synaptosomal vesicles. The reaction mixture contained in 0.1 ml: 0.15 M KCl, 0.01 M Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 10 μM $^{45}CaCl_2$ (0.02 μCi), 2 mM ATP and 15 μg synaptosomal vesicle protein. Ca^{2+} uptake in the absence of ATP and zero-time values were subtracted. The Ca^{2+} taken up by the vesicles in the presence of various concentrations of quercetin (O——O), morin (×——×) or rutin (□——□) is shown. B. The effect of flavonoids on ATP hydrolysis in synaptosomal vesicles. The reaction mixture was identical to (A) except that [γ-^{32}P]ATP (0.5 μCi/0.1 ml) was used. ATP hydrolysis in the presence of various concentrations of quercetin (O——O), morin (×——×) or rutin (□——□) is shown.

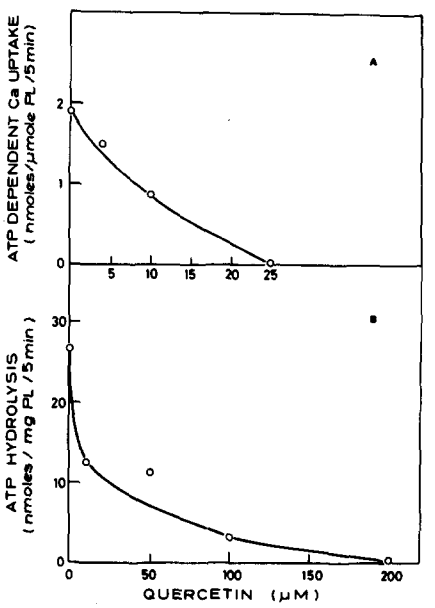

Fig. 2. A. The effect of quercetin on ATP-dependent Ca^{2+} transport in purified and reconstituted vesicles. The reaction mixture contained in 0.1 ml: reconstitution buffer (700 mM glycerol, 25 mM KCl, 25 mM ammonium acetate, 50 mM Tris, 5 mM oxalic acid, 5 mM β-mercaptoethanol (pH 7.4)), 0.1 mM $^{45}CaCl_2$ (0.2 μCi), 5.0 mM $MgCl_2$; 2 mM ATP and reconstituted vesicles (25 μg phospholipids). Vesicles were used directly from sucrose gradients. B. The effect of quercetin on ATP hydrolysis in purified and reconstituted vesicles. The reaction mixture was identical to (A) except that [γ-^{32}P]ATP was used (5 μCi/0.1 ml). The vesicles were collected after gradient centrifugation, dialysed against 10 mM Tris-HCl (pH 7.4) and lyophylized.

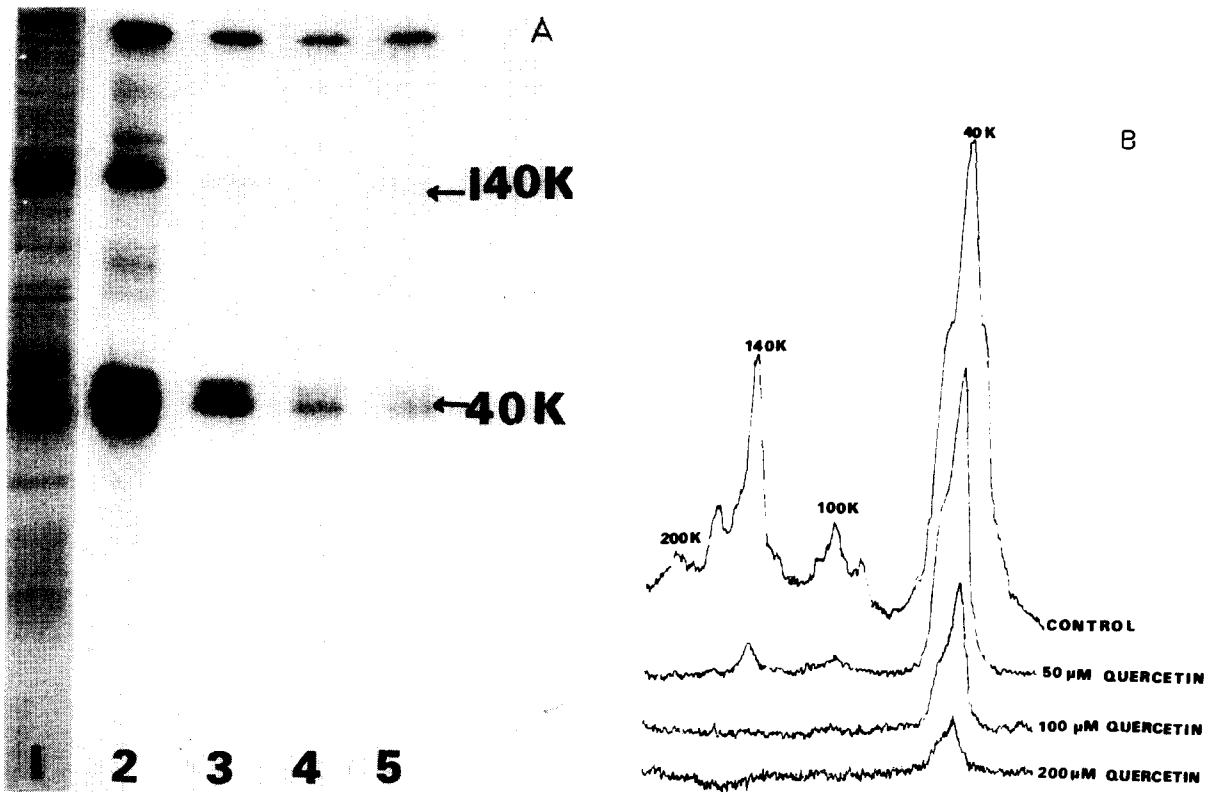

Fig. 3. The effect of quercetin on phosphorylation of synaptosomal vesicle proteins. A. Autoradiographic pattern of a SDS-polyacrylamide gel. The phosphorylation mixture contained 0.15 M KCl, 0.01 M Tris-HCl (pH 7.4), 30 μM $MgCl_2$, 2 μM [γ-^{32}P]ATP (10–20 μCi) and about 30 μg synaptosomal vesicle protein. Lane 1, Coomassie blue-stained pattern; lane 2, phosphorylated pattern obtained in the absence of quercetin; lane 3, with 50 μM quercetin added to the vesicles prior to the phosphorylation; lane 4, with 100 μM quercetin; and lane 5, with 200 μM quercetin. B. The densitometric trace of lanes 2–5.

centration was 0.01 mM, it can be calculated that the vesicles concentrated Ca^{2+} inward at least 200-fold. When the Ca^{2+} ionophore A23187 is added to the vesicles, the calcium taken up by the vesicles is rapidly released (see for example Ref. 11 or Fig. 5).

Since the flavonoids did not inhibit the small amount of Ca^{2+} associated with the vesicles in the absence of ATP (representing either passive Ca^{2+} entry into partially permeable vesicles or binding to the membranes) we can assume that the inhibitory action of the flavonoids is directed against the Ca^{2+} pump.

The difference observed between the concentration of quercetin required to inhibit the ATP-dependent Ca^{2+} transport and the ATP hydrolysis probably reflects the fact that the synaptosomal vesicle preparation contains additional ATPases unrelated to the Ca^{2+} transport [12]. Therefore, we tested also the concentration of quercetin required to inhibit Ca^{2+} transport and ATP hydrolysis in the purified and reconstituted Ca^{2+}-transport ATPase from synaptosomal vesicles. Fig. 2 shows that here, indeed, comparable quercetin concentrations are needed to obtain similar effects. 50% inhibition of Ca^{2+} transport is obtained with 10 μM quercetin and the same inhibition of ATP hydrolysis is obtained at 20 μM quercetin.

In the $(Ca^{2+} + Mg^{2+})$ATPase of sarcoplasmic reticulum and in the $(Na^+ + K^+)$-ATPase [19,20], ion translocation is preceded by formation of a phosphorylated enzyme intermediate. Kuriki and Racker [2] found that the formation of the $(Na^+ + K^+)$-ATPase phosphoenzyme from [γ-^{32}P]ATP

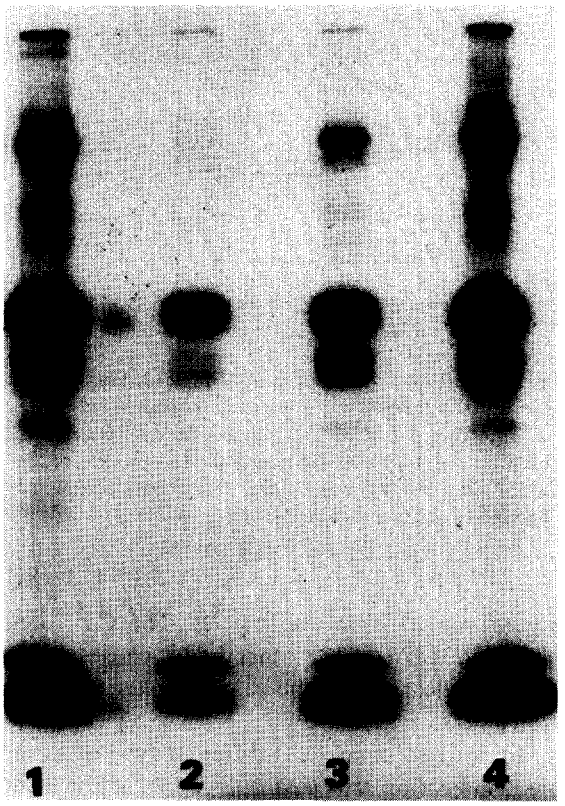

Fig. 4. The effects of quercetin, morin and rutin on phosphorylation of synaptosomal vesicle proteins. The autoradiographic picture of the SDS-polyacrylamide gel is shown. The reaction conditions are identical to Fig. 3. Lane 1 shows the control experiment in the absence of flavones; lane 2 shows the phosphorylated pattern obtained in the presence of 200 μM quercetin; lane 3, 200 μM morin; and lane 4, 200 μM rutin.

was not inhibited by quercetin, while the phosphoenzyme formation from $^{32}P_i$ (inorganic phosphate) was inhibited.

Therefore we investigated also the effect of the flavonoids on the phosphorylation of synaptosomal vesicles proteins following incubation of the membranes with [γ-^{32}P]ATP. Fig. 3A shows the SDS-gel pattern of synaptosomal vesicles incubated with [γ-^{32}P]ATP. Lane 1 shows the Coomassie blue-stained pattern of membrane proteins, lane 2 shows the autoradiography obtained of those proteins that were phosphorylated, and lanes 3, 4 and 5 show the autoradiography of identical amounts of membranes phosphorylated in the same way as in lane 2 except that 50, 100 and 200 μM quercetin was added to the membrane prior to the phosphorylation reaction. Fig. 3B shows the densitometric trace of lanes 2, 3, 4 and 5.

In native synaptosomal vesicles, several proteins undergo phosphorylation when the membrane is incubated for 20 s with [γ-^{32}P]ATP. Among them are the bands corresponding to those obtained when the purified Ca^{2+}-transport ATPase is analysed by SDS-polyacrylamide gel electrophorasis, namely those of 200, 140 and 100 kDa [12]. Some of the other membrane proteins that undergo phosphorylation under the experimental conditions used represent probably endogenous protein kinase-mediated phosphorylations.

As shown in Fig. 3, all the protein phosphorylations in the native synaptosomal vesicle membrane are inhibited by quercetin in a dose-dependent manner. Morin and rutin, on the other hand, have only slight effect on phosphorylation in synaptosomal vesicle membrane.

Fig. 4 shows the autoradiography obtained from SDS gel pattern of synaptosomal vesicle proteins phosphorylated in the presence of quercetin, morin and rutin. In lane 1 the control experiment without addition of any flavonoids to the membranes is shown. Lanes 2, 3 and 4 show the phosphorylated pattern obtained in the presence of 200 μM quercetin, morin and rutin, respectively. While quercetin has profound inhibitory effect on the phosphorylation of synaptosomal vesicle membrane proteins, morin is somewhat inhibitory at this concentration and rutin has almost no effect on the phosphorylation reaction.

The role of the membrane itself in mediating the inhibitory effects of the flavonoids

The extent of inhibition of the Ca^{2+}-transport system with the three flavonoids was highly dependent on the amount of membrane used in the reaction mixture. Table I shows the effect of 100 μM quercetin on the ATP-dependent Ca^{2+} transport system of synaptosomal vesicles with varying amounts of membrane proteins. It should be noted that identical quercetin concentration can inhibit to a different extent the amount of ATP-dependent Ca^{2+} uptake at different membrane concentrations. The higher the membrane concentration, the less inhibition is obtained.

TABLE I

THE EFFECT OF DIFFERENT AMOUNTS OF SYNAPTOSOMAL VESICLE MEMBRANE ON THE QUERCETIN-INHIBITED ATP-DEPENDENT Ca^{2+} TRANSPORT

Varying amounts of synaptosomal vesicles (lipid : protein ratio, 1.4), with or without quercetin, were incubated in a final volume of 0.1 ml medium comprising: 0.15 M KCl/0.01 M Tris-HCl (pH 7.4)/5 mM $MgCl_2$ (0.1 μCi/0.1 ml). The reaction was started by adding the mixture to the preincubated vesicles.

Synaptosomal vesicles (μg protein)	ATP-dependent Ca^{2+} transport (nmol/mg protein per 5 min)		Inhibition (%)
	control	100 μM quercetin	
8	3.11 ± 0.8	0.06 ± 0.0015	98
16	2.54 ± 1.1	0.32 ± 0.06	87
32	2.5 ± 0.6	0.75 ± 0.045	70
64	2.72 ± 0.75	1.36 ± 0.13	50
128	2.42 ± 0.6	2.1 ± 0.38	15

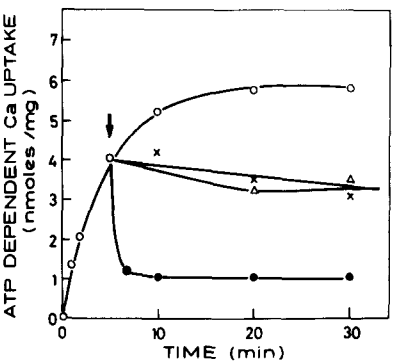

Fig. 5. The effect of quercetin on Ca^{2+} perloaded synaptosomal vesicles. Ca^{2+} loading was done under conditions identical to Fig. 1A. After 5 min of Ca^{2+} uptake (arrow), either 5 mM EDTA (× ——— ×), 100 μM quercetin (△ ——— △) or 2 μM A23187 (● ——— ●) was added. The control experiment without any additions is also shown (○ ——— ○).

The inhibitory action of quercetin is not due to a mechanism resulting in damage to the membrane structure. Fig. 5 shows an experiment in which quercetin was added to actively preloaded Ca^{2+}-containing vesicles. The efflux rate of calcium from the vesicles following quercetin addition was measured for 30 min. It can be seen that addition of quercetin at $t = 5$ min prevents further Ca^{2+} uptake by the vesicles in a way similar to the effect obtained by 5 mM EDTA. The passive efflux is not prevented from the vesicles. The Ca^{2+} ionophore A23187, on the other hand, causes an immediate release of all the calcium taken up by the vesicles, as expected.

Table II summarizes a different type of experiment indicating also that quercetin's action is not due to permanent irreversible damage to the membrane. Quercetin at two concentrations, 50 μM and 100 μM, was added to synaptosomal vesicles. ATP-dependent Ca^{2+} uptake was measured and compared to control vesicles not containing quercetin. The extent of inhibition obtained has been determined to be 70% and 100%, respectively, of the ATP-dependent Ca^{2+} uptake.

TABLE II

THE EFFECT OF LIPSOME 'WASHES' ON QUERCETIN INHIBITED SYNAPTOSOMAL VESICLES

The reaction mixture was identical to Table I; the detailed procedure for liposome treatment of quercetin-inhibited synaptosomal vesicles is described under Materials and Methods.

Quercetin (μM)	ATP-dependent Ca^{2+} uptake (nmol/mg per 5 min)	Inhibition (%)	ATP-dependent Ca^{2+} uptake after liposome treatment (nmol/mg per 5 min)	Inhibition (%)
–	2.65	–	1.75	–
50	0.795	70	1.7	3
100	0.0	100	1.49	15

At the end of the 'uptake period', liposomes (phosphatidylcholine vesicles formed by ultrasonic treatment) were added to the inhibited and control synaptosomal vesicles for 20 min, this being followed by the separation of synaptosomal vesicles from the reaction mixture by centrifugation. The ATP-dependent Ca^{2+} uptake was measured again. It can be seen that liposome treatment restores almost completely the ability of the vesicles to take up calcium. 'Washing' synaptosomal vesicles with liposomes causes some decrease in their activity. This decrease, however, is identical for both control and quercetin-treated vesicles.

Comparison of physical properties of quercetin, morin and rutin

The three flavonoids used in this work have different inhibitory potencies. Quercetin is the most potent of the three flavonoids used. It inhibited Ca^{2+} uptake, ATP hydrolysis and phosphorylation at very low concentrations. Morin and rutin were both much less efficient as inhibitors of Ca^{2+} transport, ATP hydrolysis and phosphorylation. In order to test whether the difference observed in their relative potencies as inhibitors could be explained by their different affinities for the membrane lipid phase we have done three different types of experiment:

(1) determination of the partition of quercetin, morin and rutin between water and olive oil;

(2) measurement of their uptake by synaptosomal vesicles;

(3) measurement of the uptake of the three flavonoids by phosphatidylcholine liposomes.

Fig. 6 shows an experiment where each of the flavones was added to a mixture of water and olive oil. After proper mixing, the phases were allowed to separate and the absorbance at 375 nm (for quercetin and rutin) or 390 nm (for morin) was determined of each fraction collected from the bottom. It can be seen that quercetin is found almost exclusively in the olive oil and rutin in the water phase, and that morin is evenly distributed between both phases.

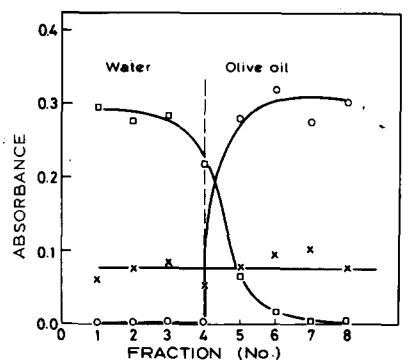

Fig. 6. The partitioning of the flavonoids between aqueous phase and olive oil. Quercetin (○——○), morin (×——×) and rutin (□——□) at a concentration of 100 μM were added to a biphasic solution of olive oil and 0.15 M KCl/0.01 M Tris-HCl (pH 7.4). Following vigorous mixing, the phases were separated by centrifugation and aliquots of 250 μl were collected from the bottom. The flavone concentration in each fraction was measured spectrophotometrically at its maximal extinction (375 nm for quercetin and rutin, 390 nm for morin).

TABLE III

UPTAKE OF FLAVONOIDS BY SYNAPTOSOMAL VESICLES

Synaptosomal vesicles were preincubated with 100 μM flavone and added ro a reaction mixture comprising 0.15 M KCl/0.01 M Tris-HCl (pH 7.4)/5 mM $MgCl_2$. The final volume was 2 ml. The fluorescence was determined before and after the membranes were separated by filtration through 0.45 μm nitrocellulose filters from the reaction mixture. Fluorescence: excitation: quercetin, 438 nm; morin and rutin, 432 nm; emission: quercetin; 530, 540; rutin, 515, 525 (nm), morin, 545, 535 (nm). Values expressed as area of the peak at maximum emission.

Flavone (100 μM)	Flavone fluorescence with membranes	Flavone fluorescence after membrane removal	Flavone taken up (%)
Quercetin	89.2	6.32	93
Morin	105.7	93.8	11
Rutin	13.7	10.9	17

Table III shows the uptake of the flavonoids by the synaptosomal vesicle membranes during the reaction of Ca^{2+} uptake. At the end of the uptake reaction, the membranes were removed by filtration on nitrocellulose filters; the area of the peak of fluorescence at maximal emission was determined in the filtrate and compared to that obtained in the presence of the membranes. It can be seen that quercetin is taken up almost com-

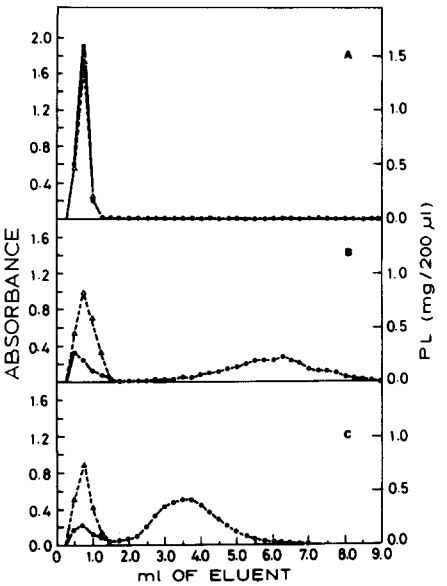

Fig. 7. The uptake of flavonoids by phosphatidylcholine vesicles. Uptake of quercetin (A), morin (B) and rutin (C) at a concentration of 100 μM by phosphatidylcholine liposomes was measured following a 20 min preincubation period in a reaction mixture identical to the Ca^{2+} liposomes were separated from the reaction mixture by filtration through a Sephadex G-50 (fine) column. The phospholipid phosphate (△———△) and flavone concentration (●———●) in each fraction was determined.

pletely by the vesicles (93% uptake), while morin and rutin are taken up only very poorly by the membranes (11% and 17% respectively).

Similar results were obtained when phosphatidylcholine vesicles (without any proteins) were incubated with the three flavonoids (Fig. 7). The liposomes were separated from the reaction mixture by passage through Sephadex G-50 (fine) column. The liposomes were not retained by the column, while the 'free' flavonoids (not associated with the liposomes) were retained by the column, and eluted only after washing the column with several column volumes of buffer. In this experiment, also, as with native synaptosomal vesicles, quercetin was taken up almost completely by the liposomes (Fig. 7A) and therefore it has been recovered in the void volume (the phospholipid peak coincided with the absorbance). Only small amounts of rutin (Fig. 7B) and morin (Fig. 7C) are eluted from the column with the liposomes. Most of the rutin and morin is eluted from the column following several (about four) column volumes.

TABLE IV

THE EFFECT OF FLAVONE ON HISTONE PHOSPHORYLATION

Histone phosphorylation was measured in 0.2 ml reaction mixture containing 30 mM Tris-HCl (pH 7.4) 5 mM $MgCl_2$, 50 μg histone and either 10 units of bovine heart protein kinase (A) or 200 μg of adrenal cortex protein kinase (B). The histone was preincubated for 20 min at 37°C with or without the flavonoids and the reaction was started by adding 50 μM [γ-^{32}P]ATP – about 15 μCi.

Flavone (200 μM)	Phosphorylation (pmol P_i/mg histone per 5 min)	Inhibition (%)
A –	1072	–
Quercetin	116	89
Morin	331	69.7
Rutin	324	69.2
B –	900	–
Quercetin	358	61
Morin	458	50
Rutin	504	44

Additional support for the hypothesis that the inhibitory action of the flavonoids and their different potencies are related to their solubility in the membrane lipid phase comes from the following experiment. The inhibitory action of quercetin, morin and rutin was tested on a soluble system, namely histone phosphorylation by protein kinase.

Table IV shows the results obtained by adding quercetin, morin and rutin to protein kinase-mediated phosphorylation of histones. Two different protein kinases have been used. In both systems which are not part of any membranous structure, but soluble proteins, all three flavonoids inhibited phosphorylation to a similar extent.

Discussion

Quercetin, morin and rutin – the three plant flavonoids used in these studies – represent three structurally similar compounds: quercetin and morin differ in the position of one hydroxyl group (2′ instead of 3′) in the same ring, while rutin is the rutinose (6-*O*-2-rhamnosyl D-glucose) derivative of quercetin.

All three flavonoids used inhibited ATP-dependent Ca^{2+} transport (Fig. 1) in synaptosomal vesicles. Quercetin was the most efficient inhibitor of the Ca^{2+} transport system, while both morin and rutin were much less efficient as inhibitor of Ca^{2+} transport (Fig. 1A) and ATP hydrolysis (Fig. 1B).

In native synaptosomal vesicles, much higher quercetin concentrations were required to inhibit ATP hydrolysis than Ca^{2+} transport. One possible explanation of these results could be related to the fact that native vesicles contain ATPases unrelated to the Ca^{2+} transport [12], but probably interacting with quercetin (and other flavonoids) as well. Another explanation could be that the Ca^{2+}-transporting site and the ATPase have different affinities for the flavonoids.

The first possibility is strongly supported by the fact that the ratio of Ca^{2+} transport to ATP hydrolysis in native synaptosomal vesicles is very small (0.06), even in the presence of oxalate (a Ca^{2+} trapping agent), while in reconstituted and purified vesicles this ratio is 0.95, [12]. To demonstrate this directly, quercetin was added to purified and reconstituted synaptosomal vesicle Ca^{2+}-ATPase (Fig. 2A B) and its inhibitory effects were measured. Here, indeed, comparable quercetin concentrations inhibited Ca^{2+} transport and ATP hydrolysis.

Thus, unlike $(Na^+ + K^+)$-ATPase [7,8], in the case of which Rb^+ transport was unaffected by quercetin while ATP hydrolysis was inhibited, in synaptosomal vesicles, as in the inside-out red blood cell ghost Ca^{2+} pump [6], both Ca^{2+} transport and ATP hydrolysis were affected to similar extent.

Beside inhibition of Ca^{2+} transport and ATP hydrolysis, the flavonoids also inhibited phosphorylation of synaptosomal vesicle membrane proteins by ATP. The inhibition of phosphorylation was dose-dependent (Fig. 3) and with 200 μM quercetin almost all phosphorylatable proteins in the synaptosomal vesicle membrane were affected. It was interesting to note that not only proteins related to ATP-dependent Ca^{2+} transport were affected. The purified Ca^{2+}-transporting system contains three proteins, as analysed on SDS containing polyacrylamide gels, corresponding to 94, 140 and 200 kDa [12]. These three proteins are probably self-phosphorylated as part of the reaction mechanism of ATP hydrolysis. However, in native synaptosomal vesicles many more bands are phosphorylated upon addition of [γ-^{32}P]ATP and all these are inhibited by addition of quercetin.

One attractive possibility was that these phosphorylated proteins were the reaction products of endogenous protein kinase. If this were the case, the flavonoids would have exerted their inhibitory action not only via ATPase-mediated self-phosphorylations but by protein kinase-mediated protein phosphorylation as well. To test this hypothesis directly, the effect of the flavonoids on histone phosphorylation by protein kinase was measured. Table IV shows the results of this experiment. Quercetin, morin and rutin at a concentration of 200 μM all considerably inhibited histone phosphorylation.

Very little is known about the mechanism of action of these flavonoids. The results of the experiments presented here can rule out their inhibitory action being caused by some permanent damage to the membrane.
(1) Addition of quercetin to Ca^{2+}-preloaded synaptosomal vesicles did not result in complete loss of Ca^{2+} from the vesicles, like that observed upon addition of the Ca^{2+} ionophore A23187 (Fig. 5). The only effect of the added flavonoid was to prevent further Ca^{2+} uptake, as with the divalent cation chelator EDTA. These results are in agreement with the observation of Shoshan et al. [5] that in sarcoplasmic reticulum passive efflux of calcium from the vesicles was unaffected upon addition of quercetin. (2) The inhibition of Ca^{2+} transport caused by addition of quercetin to synaptosomal vesicles can be reversed easily by 'washing' the inhibited vesicles with phosphatidylcholine liposomes (Table II). This finding also supports the hypothesis that the flavonoids probably do not penetrate into the vesicles, but remain associated with the membrane.

In order to account for the profound differences in the potency of the three flavonoids tested, we have investigated the possibility that the relative affinity of the flavonoids for the membrane lipid phase played a major role. Three types of experiment were performed: (1) the partitioning of the three flavonoids between water and olive oil was measured; (2) the uptake of the flavonoids by

native synaptosomal vesicles was determined, as was (3) the uptake of these flavonoids by phosphatidylcholine liposomes. All three experiments pointed unequivocally to the fact that quercetin was the most lipid-soluble flavonoid of the three tested. It partitioned exclusively in the olive oil, over 90% of it was taken up by both native membranes and liposomes from the reaction medium. Morin and rutin both had much smaller affinity for the lipid phase. Thus, the relative potency of the three flavonoids tested did not result from some specific difference between them, but was strongly dependent on their possibility to interact with the membrane. The strongest support for this hypothesis can be obtained from the results shown in Table IV, in which the effect of three flavonoids was tested on a soluble protein. All three flavonoids had comparable inhibitory effects on histone phosphorylation by the two protein kinases tested.

Acknowledgements

We would like to thank Dr. R. Rahamimoff and Dr. S. Schuldiner for critically reading this manuscript and Mrs. Na'ama Nesher for her unfailing help in preparing it. This work was supported in part by the Muscular Dystrophy Association, N.Y., and the United States-Israel Binational Science Foundation. Part of this work was submitted by A.B. to the Hebrew University Medical School as his M.Sc. Thesis.

References

1 Carpenedo, F., Bortignon, C., Bruni, A. and Santi, R. (1969) Biochem. Pharmac. 18, 1495–1500
2 Kuriki, Y. and Racker, E. (1976) Biochemistry 15, 4951–4955
3 Lang, D. and Racker, E. (1974) Biochim. Biophys. Acta 333, 180–186
4 Gompert, B.D. and Fewtrell, C.M. (1977) Nature 265, 635–636
5 Shoshan, V., Campbell, K.D., MacLennan, D.H., Frodis, W. and Brit, B.A. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 4435–4438
6 Wuthrich, A. and Schatzman, H.J. (1980) Cell Calcium 1, 21–35
7 Suolinna, E.M., Lang, D. and Racker, E. (1974) J. Natl. Cancer Inst. 53, 1515–1519
8 Suolinna, E.M., Buchsbaum, R.N. and Racker, E. (1975) Cancer Res. 35, 1865–1872
9 Rahamimoff, H. and Abramovitz, E. (1978) FEBS Lett. 89, 223–225
10 Rahamimoff, H. and Abramovitz, E. (1978) FEBS Lett. 92, 163–167
11 Rahamimoff, H., Abramovitz, E., Papzian, D., Goldin, M.S. and Spanier, R. (1980) J. Physiol. Paris 76, 487–495
12 Papazian, D., Rahamimoff, H. and Goldin, M.S. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3708–3712
13 Rahamimoff, R. (1980) in The Role of Intercellular Signals Navigation, Encounter, Outcome (Nicholls, J., ed.), pp. 15–40, Dahlem
14 Gasko, O.D., Knowles, A.F., Shertzer, H.G., Suolinna, E.M. and Racker, E. (1976) Anal. Biochem. 72, 57–65
15 Goldin, M.S. (1977) J. Biol. Chem. 252, 5630–5642
16 Laemmli, U.K. (1970) Nature 227, 680
17 Kuo, J.F. and Greengard, P. (1970) J. Biol. Chem. 245, 4067–4073
18 Barenholtz, Y., Gibbs, D., Litman, B.J., Goll, J., Thompson, T.E. and Carlson, F.D. (1977) Biochemistry 16, 2806–2810
19 Tada, M., Yamamoto, T. and Tonomura, Y. (1978) Physiol. Rev. 58, 1–79
20 Post, R.L., Hegyvary, C. and Kume, S. (1972) J. Biol. Chem. 247, 6530–6540