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ELEVATION OF CYCLIC AMP LEVEL IN EHRLICH ASCITES TUMOR CELLS BY QUERCETIN

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Abstract—Quercetin, a flavonoid known to cause inhibition of ATPase activity and cell growth *in vitro*, increases cyclic AMP levels in Ehrlich ascites tumor cells. The possibility of interrelationship between cyclic AMP metabolism and $(Na^+ + K^+)$ATPase activity is discussed.

Many investigations suggest that cyclic AMP is involved in the control of cell growth. In several types of malignant cells a low level of cyclic AMP was found and it was suggested that this low level is responsible for the loss of contact inhibition and uncontrolled growth of tumor cells [1–5].

Racker and coworkers proposed recently [6–8], that the increase in ATPase activity in different pump systems, may be the reason for the elevation of aerobic glycolysis in various malignant cells. Kasarov and Friedman claim that there exists an interrelationship between cyclic AMP level and $(Na^+ + K^+)$-ATPase activity in transformed fibroblasts [9]. Furthermore, it was shown that inhibition of the $(Na^+ + K^+)$-ATPase activity by ouabain in guinea pig cerebral cortex increases indirectly cyclic AMP levels [10]. It was also suggested, that cyclic AMP inhibits $(Na^+ + K^+)$-ATPase activity in rat liver plasma membranes [11, 12].

Quercetin (3,3',4',5,7 pentahydroxy flavone) inhibits the high aerobic glycolysis in tumor cells. It was suggested that this inhibition is due to the effect of quercetin on different ATPase systems [6, 7]. Quercetin is also known as a potent inhibitor of protein synthesis [13] and cell growth [7]. On the basis of the above observations, it is of interest to find out whether quercetin has any influence on cyclic AMP levels in tumor cells. The present experiments were done with Ehrlich ascites tumor cells in which the high glycolysis level was proposed to be regulated by $(Na^+ + K^+)$-ATPase [8, 14, 15].

MATERIALS AND METHODS

Ehrlich ascites cells were maintained in ICR male mice harvested 7 days after transplantation and washed as previously described [8]. The medium used for the cell washing and for the determinations of cyclic AMP contained 50 mM sodium Tricine [*N*-Tris (hydroxymethyl)-methylglycine], 4 mM sodium phosphate buffer pH 7.4, 100 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$ and 0.5 mM $CaCl_2$. Cells were washed twice with the above medium and incubated at 30° with and without quercetin or ouabain, at the concentrations indicated in each experiment. Since quercetin was dissolved in dimethyl sulfoxide (DMSO), this substance was added to each flask, that did not contain quercetin, as control, at concentrations of 5 μl/ml incubation medium. Incubation was terminated by adding 0.8 ml of the incubated cell suspension to 0.2 ml 0.5 M acetate buffer pH 4 and boiling for 3 min. Cyclic AMP was determined according to Gilman [16] in the deproteinized extract.

Plasma membranes of Ehrlich ascites tumor cells were prepared by the use of the aqueous two phase dextran polyethylene glycol system as described by Brunette and Till [17]. By using this procedure we obtained at least a 10-fold purification of the $(Na^+ + K^+)$-ATPase activity in the plasma membrane preparation compared to the crude homogenate. The specific activities were recorded as 0.35 and 0.03 μmoles/mg/min in plasma membrane preparations and crude homogenate respectively. Furthermore, ATPase activity in plasma membrane preparations was inhibited by 1 mM ouabain (up to 70 per cent) but slightly affected by azide, a mitochondrial ATPase inhibitor (less than 10 per cent inhibition). The ATPase activity in the plasma membranes was determined by the release of labeled inorganic phosphate from [γ-^{32}P]-ATP as described elsewhere [18]. The assay was performed in incubation medium which contained 50 mM sodium tricine 4 mM sodium phosphate buffer, pH = 7.4, 100 mM NaCl, 5 mM KCl and 2 mM $MgCl_2$ using 0.1 mg protein of plasma membranes for each assay. $CaCl_2$ was omitted from the incubation medium in order to get maximal activity of the $(Na^+ + K^+)$-ATPase [19]. The reaction was started by addition of 2 mM [γ-^{32}P]-ATP and after 10 min incubation at 30°, was stopped by addition of 50 μl/ml 40% w/v TCA. ATP levels were measured as described [20]. Protein was measured by the method of Lowry *et al.* [21].

RESULTS AND DISCUSSION

Cyclic AMP elevation was shown to be dependent on quercetin concentration (Table 1), as well as on the incubation duration (Fig. 1). A maximal level was reached in the presence of 10^{-4} M quercetin (Table 1). When this concentration was used. the maximal level was reached about eight min after quercetin was added (Fig. 1). The change in the amount of cyclic AMP as a result of incubation with quercetin is also a function of cell concentration as indicated in Fig. 2.

Table 1. Cyclic AMP level in Ehrlich ascites cells (3 mg protein/ml) incubated for 10 min with various concentrations of quercetin*

Additions	Cyclic AMP pmoles per mg protein
	Mean ± S.E
None	3.6 ± 1.4
25 μM Quercetin	4.6 ± 1.2
50 μM Quercetin	6.1 ± 1.4
100 μM Quercetin	10.7 ± 1.8
250 μM Quercetin	10.3 ± 1.8

* The results are mean of 16 samples taken from four animals

The findings showing that the elevation of cyclic AMP level is a function of incubation time, quercetin concentration and cell concentration, suggest that quercetin may exert its effect through the enzymes which control cyclic AMP metabolism, namely adenyl cyclase and phosphodiesterase. Cyclic AMP level was found to increase also in cells treated with ouabain, a specific inhibitor of $(Na^+ + K^+)$-ATPase (Table 2). At concentration of 10^{-3} M, ouabain induced a 2.3-fold increase of cyclic AMP level, while quercetin (10^{-4} M) effected a 3.5-fold increase under same conditions. At the same concentrations of quercetin and ouabain, inhibition of $(Na^+ + K^+)$-ATPase activity in isolated plasma membranes was observed (Fig. 3). Considering the molar concentration for $(Na^+ + K^+)$-ATPase inhibition, quercetin was found to be a 10-fold more potent inhibitor than ouabain in these systems.

Since quercetin, like ouabain, is shown to inhibit $(Na^+ + K^+)$-ATPase activity, a casual relationship between the activity of this enzyme and cyclic AMP level in Ehrlich ascites cells may be postulated, thus supporting similar claims in other malignant cells [9]. The reduction of $(Na^+ + K^+)$-ATPase activity of liver plasma membranes by cyclic AMP (10^{-5} M) [11, 12], further indicates a possible relationship between the enzymes responsible for cyclic AMP metabolism and $(Na^+ + K^+)$-ATPase activity. Alternatively, $(Na^+ + K^+)$-ATPase activity and cyclic AMP level, may be interrelated due to the fact that ATP is a common substrate in both systems. According to this hypothesis, the elevation of cyclic AMP level by quercetin results from the possible accumulation of ATP caused by the reduction of ATP utilization by the $(Na^+ + K^+)$-pump system. The data shown in Table 2 exclude such a proposition since similar levels of ATP were recorded in the absence and presence of both quercetin and ouabain.

Table 2. The effect of 10 min incubation with ouabain and quercetin on cyclic AMP and ATP levels in Ehrlich ascites tumor cells (3 mg protein/ml)*

Additions	Cyclic AMP pmoles per mg protein	ATP nmoles per mg protein
	Mean ± S.E.	Mean ± S.E.
None	3.8 ± 1.0	18.3 ± 2.3
10^{-3} M Ouabain	8.7 ± 1.6	15.7 ± 0.9
10^{-4} M Quercetin	13.4 ± 2.4	18.9 ± 4.2

* Number of determinations: 5.

The increase in $(Na^+ + K^+)$-ATPase activity found in transformed cells [22], may be related to the control of growth by Na^+ and K^+ ions [23], as

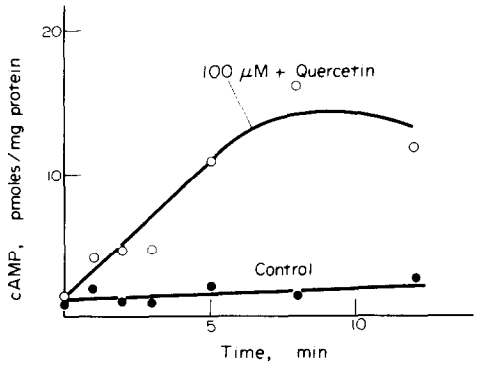

Fig. 1. Cyclic AMP level in Ehrlich ascites cells (3 mg protein/ml) incubated with and without quercetin for different lengths of time. The results represent one typical experiment out of 4 experiments.

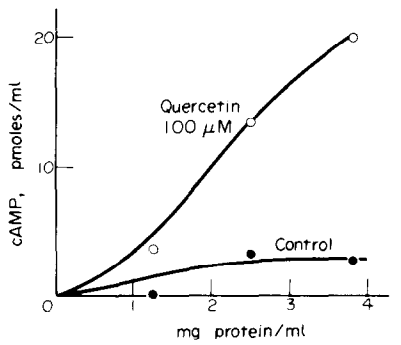

Fig. 2. Cyclic AMP level in different concentrations of Ehrlich ascites cells incubated for 10 min with and without quercetin (10^{-4} M). The results are means of 8 samples taken from 2 animals.

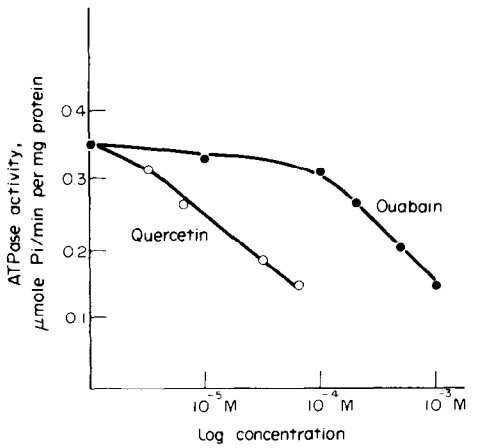

Fig. 3. The effect of quercetin and ouabain on ATPase activity in plasma membranes. The ATPase activity was determined as described in materials and methods. The data represents the mean of 4 different preparations with S.E. of 8–15 per cent.

$(Na^+ + K^+)$-ATPase plays a critical role in $(Na^+ + K^+)$-pump system [24]. Recently it was suggested, that in Ehrlich ascites tumor cells quercetin rectifies the high glycolytic rate by repairing the assumed defective control mechanism of the $(Na^+ + K^+)$-pump system [6, 7].

The results in this study may indicate the existence of interrelationship between $(Na^+ + K^+)$-ATPase activity and cyclic AMP level in Ehrlich ascites tumor cells.

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