Inhibition of virus multiplication and alteration of cyclic AMP level in cell cultures by flavonoids

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Summary. The inhibitory effect of four flavonoid compounds on virus multiplication and their influence on the intracellular cyclic AMP (cAMP) level were studied in cell cultures. Quercetin and quercitrin reduced the yields of *Human (alpha) herpesvirus* 1 (HSV-1) and *Suid (alpha) herpesvirus* 1 (pseudorabies virus), but hesperidin and rutin had no effect. Further, quercetin and quercitrin elevated the intracellular level of cAMP, whereas hesperidin and rutin did not alter the cAMP level. Both antiviral activity and cAMP-enhancing effect were dependent on the concentrations of the flavonoids, and these effects turned out to be parallel.
This study suggests that a relation exists between the antiviral effect and the cAMP-enhancing activity of flavonoids.
Key words. flavonoids; cyclic AMP-enhancing activity; *human (alpha) herpesvirus* 1; *Suid (alpha) herpesvirus* 1.

The flavonoids are widely distributed and important compounds in nature; they exhibit numerous biological activities and some of them have already been applied in human therapy[2]. The antiviral effects of flavonoids in animals were described first by Cutting et al.[3,4]. Our earlier data demonstrated the antiviral activities of different flavonoids in cell cultures[5,6]. Later, it was also shown that quercetin and morin exert significant protective effects against Mengo virus-induced encephalitis in mice[7]. Various enzyme activities are also affected by flavonoid compounds[2]. It has recently been reported that quercetin raises the cyclic AMP (cAMP) level in Ehrlich ascites tumor cells[8]. Flavonoids have also been described as potent inhibitors of cAMP phosphodiesterase, which is responsible for the breakdown of cAMP[9,10]. In addition, it has been shown that cAMP-enhancers, such as dibutyryl cAMP, inhibit the multiplication of herpes simplex viruses[11-13]. These observations suggested that it would be worthwhile to study in parallel the antiviral activities of four flavonoids and their effects on the intracellular cAMP level. In the present paper, data are presented which point to a relation between these effects of the flavonoids.

Materials and methods. Chemicals. Quercetin (Merck), quercitrin (Calbiochem), hesperidin (Calbiochem) and rutin (Serva) were commercial products. Stock solutions were prepared in dimethylsulfoxide, and culture medium was used for further dilutions.

Viruses and cell cultures. The HEp-2 cells were cultured in Eagle's basal medium as modified by Macpherson and Stoker[14], supplemented with 5% calf serum and 10% tryptose phosphate broth. Chick embryo fibroblast (CEF) cells were maintained in Gey's solution, containing 4% pH 7.6 Tris-HCl buffer, 5% calf serum and 0.25% lactalbumin hydrolysate. A strain of HSV-1 isolated from human conjunctiva in our laboratory was used. HSV-1 was grown in HEp-2 cells and the infectivity was measured in the same cells by the dilution method in microtitre trays (Linbro, Greiner). The infective titre of HSV-1 was expressed as $TCID_{50}$, calculated by the formula of Reed and Muench[15]. Pseudorabies virus was isolated from pig brain as previously described[16], and propagated in CEF cells. The infectivity of pseudorabies virus was determined by the plaque method.

Assay of intracellular cAMP level. Basically the procedure of Degré and Rollag[17], was followed with a minor modification. For cAMP assay, cells were grown in 60-mm diameter glass Petri dishes. The monolayers were treated with the various flavonoids at 37 °C for 15 min in the cell medium. After incubation the cells were scraped off with a rubber policeman. Parallel cultures were trypsinized for determination of cell counts. The cells were suspended in acetate buffer (10 mM, pH 6.4) and boiled for 2 min, sonicated for 15 s, and spun down in an Eppendorf centrifuge for 5 min (12.5 K × g, at 4 °C) to remove the cell debris. The cAMP content of the samples was assayed with a cAMP Kit (Amersham). The values were expressed in picomoles cAMP per 10^7 cells, representing three samples, each tested twice in parallel.

Determination of antiviral activity. The inhibitory effects of the compounds were determined by the yield reduction method. The HEp-2 cells (2×10^6) were seeded in 50-mm diameter glass Petri dishes and the monolayers were infected with $10^{4.5}$ $TCID_{50}$ of HSV-1. After adsorption for 1 h at 37 °C, the inoculum was removed and the cultures were washed twice with Hanks' solution and supplied with Eagle's medium containing the drugs at

Effects of flavonoids on virus yields

Flavonoid	Concentration (μM)	Reduction of yields* of HSV-1 (log_{10})	pseudorabies virus (log_{10})
Quercetin	250	2.1	2.0
	150	2.0	1.4
	100	0.0	0.7
	10	0.0	0.0
Quercitrin	500	2.1	2.6
	250	0.9	1.3
	100	0.0	0.7
	10	—	0.0
Hesperidin	500	0.2	0.3
	250	0.0	0.2
	100	0.0	0.0
Rutin	750	0.0	0.2
	500	0.0	0.2
	100	0.0	0.2

* Results are mean values of two experiments.

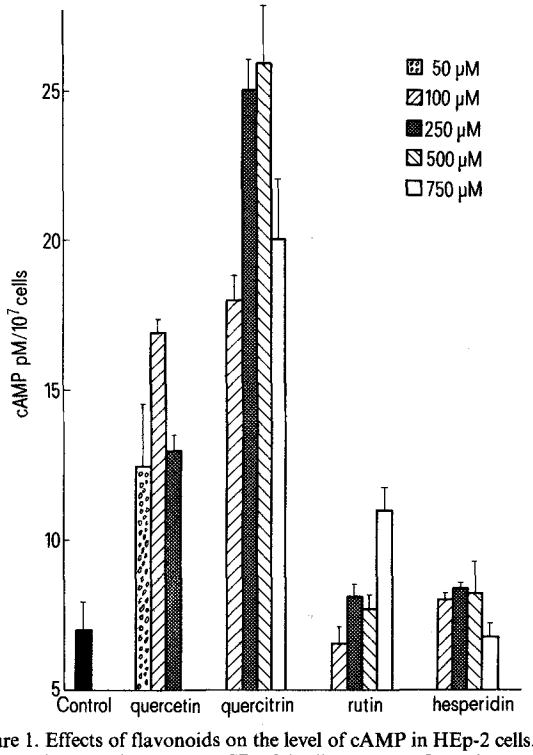

Figure 1. Effects of flavonoids on the level of cAMP in HEp-2 cells. The cAMP values are the means ± SD of duplicate assays from three experiments. Control cultures were incubated without flavonoids.

different concentrations. Control cultures were treated with drug-free medium. After incubation for 20 h, the cultures were frozen and thawed. The cell debris was removed by low-speed centrifugation and the supernatant was titrated by the dilution method. The antiviral effects of the compounds against pseudorabies virus were investigated in CEF cells using the yield reduction test too. CEF cells (1.2×10^7) were seeded in 50-mm diameter glass Petri dishes. The confluent monolayers of cells were infected with 500 plaque-forming units (PFU) of pseudorabies virus. After the adsorption period the inoculum was removed, the cultures were washed twice with Hanks' solution, and the cells were then fed with culture medium with or without drugs. The incubation was continued for 20 h at 37 °C and the procedure for the determination of virus yields was the same as in the case of HSV-1. The virus content was determined by the plaque method and the virus titre was expressed as PFU. For evaluation of the virus inhibitory effect, the virus titres obtained in the presence of compounds were compared with those for the untreated infected controls.

Virucidal effect of flavonoids. The virus particles were also treated with different flavonoids. Equal volumes of flavonoid solutions prepared in Eagle's medium containing 5% calf serum and the virus suspension were mixed and incubated at room temperature for 2 h. After the incubation period the virus titers were determined by the dilution method or by the plaque method, depending on the virus used.

Cytotoxicity of flavonoids. The CEF cells and HEp-2 cells were cultured in the presence of serially diluted compounds for 24 h at 37 °C. The cells were examined by light microscope and compared with the untreated control cultures.

Results and discussion. No visible alteration in cell morphology was observed by microscopic examination at the concentrations applied.

The intracellular level of cAMP was determined at different concentrations of compounds after treatment for 15 min. Quercetin and quercitrin enhanced the cAMP level in HEp-2 cells while hesperidin and rutin were without effect. The enhancing activity was dependent on the flavonoid concentration (fig. 1). Elevation of the cAMP level could also be observed in the case of CEF cells treated with quercetin and quercitrin, but hesperidin and rutin were again found to be ineffective (fig. 2). Maximum intracellular levels of cAMP were attained in the presence of 250–500 µm quercetin or 500–750 µM quercitrin.

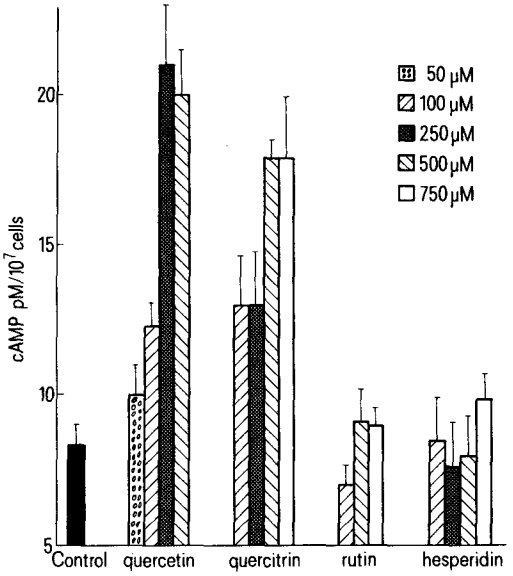

Figure 2. Effects of flavonoids on the level of cAMP in CEF cells. The cAMP values are the means ± SD of duplicate assays from three experiments. Control cultures were incubated in flavonoid-free media.

The effects of the four flavonoids on HSV-1 and pseudorabies virus multiplication were determined. The HSV-1 yield was decreased by quercetin and quercitrin, but not influenced by hesperidin and rutin (table).

Quercetin and quercitrin also showed inhibitory effects on pseudorabies virus multiplication, whereas hesperidin and rutin had no such activity (table).

These results showed that quercetin and quercitrin displayed antiviral effects against the two herpesviruses tested, while hesperidin and rutin were without effect. Flavonoids were not found to inactivate virus particles under the experimental conditions described above.

Flavonoids are naturally occurring compounds which have many important biochemical effects, and some of them have been applied in human therapy, as described in the review by Havsteen[2]. The in vivo antiviral effects of flavonoids have been described previously[3,4] and we found that some flavonoids are effective in vitro, mainly against herpesviruses[5,6]. It has also been shown that quercetin and morin exert significant protective effects against Mengo virus in mice[7]. 4',5-Dihydroxy-3,3',7-trimethoxyflavone (Ro 09-0179) has recently been found to inhibit selectively the replication of human picornaviruses[18].

The mechanism of the antiviral activity of the flavonoids is unknown. Ishitsuka et al.[18] suggested that the flavone derivative tested, Ro 09-0179, might prevent viral uncoating. It is possible that the enzymes necessary for viral uncoating are inhibited by flavonoids[2]. Quercetin has recently been reported to raise the intracellular cyclic AMP level in Ehrlich ascites tumor cells[8], and the flavonoids inhibit the cAMP phosphodiesterase responsible for the breakdown of cAMP[9,10]. Quercetin and quercitrin are potent inhibitors of cAMP phosphodiesterase[10]. Some derivatives of cAMP have been proved to have an antiviral effect[11–13,19]. Our observation that only those flavonoids able to enhance the intracellular cAMP level display antiviral effects suggests a relation between the antiviral effect and the increase in cAMP level.

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2 Havsteen, B., Biochem. Pharmac. *32* (1983) 1141.
3 Cutting, W.C., Dreisbach, R.H., and Neff, B.J., Stanford med. Bull. *7* (1949) 137.
4 Cutting, W.C., Dreisbach, R.H., and Matsushima, F., Stanford med. Bull. *11* (1953) 227.
5 Bakay, M., Mucsi, I., Béládi, I., and Gábor, M., Acta microbiol. Acad. Sci. hung. *15* (1968) 223.
6 Béládi, I., Pusztai, R., Mucsi, I., Bakay, M., and Gábor, M., Ann. N.Y. Acad. Sci. *284* (1977) 358.
7 Veckenstedt, A., Béládi, I., and Mucsi, I., Arch. Virol. *57* (1978) 255.
8 Graziani, Y., and Chayoth, R., Biochem. Pharmac. *26* (1977) 1259.
9 Beretz, A., Anton, R., and Stoclet, J.C., Experientia *34* (1978) 1054.
10 Ferrel, J.E. Jr, Chang Sing, P.B.G., Loew, G., King, R., Mansour, J.M., and Mansour, T., Molec. Pharmac. *16* (1979) 556.
11 Stanwick, T.L., Anderson, R.W., and Nahmias, A.J., Infect. Immun. *18* (1977) 342.
12 Maassab, H.F., and Soltysiak, R.M., Ann. N.Y. Acad. Sci. *284* (1977) 375.
13 Stanwick, T.L., and Nahmias, A.J., Arch. Virol. *72* (1982) 247.
14 Macpherson, I., and Stoker, M., Virology *16* (1962) 147.
15 Reed, L.J., and Muench, H.A., Am. J. Hyg. *27* (1938) 493.
16 Cserey-Pechány, É., Béládi, I., and Ivánovics, G., Acta physiol. hung. *2* (1951) 229.
17 Degré, M., and Rollag, M., J. Interferon Res. *2* (1982) 151.
18 Ishitsuka, H., Ohsawa, C., Ohiwa, T., Umeda, I., and Suhara, Y., Antimicrob. Ag. Chemother. *22* (1982) 611.
19 Biron, K.K., and Raska, K. Jr, Virology *56* (1973) 383.

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