Biochemical Pharmacology, Vol. 31, No. 7, pp. 1357–1361, 1982.
Printed in Great Britain.

0006-2952/82/071357-05 $03.00/0

SELECTIVE INHIBITION OF A CYCLIC NUCLEOTIDE INDEPENDENT PROTEIN KINASE (G TYPE CASEIN KINASE) BY QUERCETIN AND RELATED POLYPHENOLS

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(*Received* 23 *July* 1981; *accepted* 28 *October* 1981)

Abstract—The effect of quercetin and a number of structurally related phenolic compounds upon the activity of three different purified protein kinases was examined. Whereas the catalytic subunit of a cyclic AMP-dependent protein kinase and an A type (using only ATP) cyclic nucleotide-independent casein kinase (CKA) were not affected, a G type (using GTP as well as ATP) casein kinase (CKG) was selectively inhibited by several bioflavonoid structures. Kinetic studies showed that quercetin behaved as a competitive inhibitor toward the nucleotidic substrate and exhibited a high affinity for the ATP ($K_i = 0.75\ \mu M$) and GTP ($K_i = 0.22\ \mu M$) site of the enzyme. Considering the CKG inhibitory potency of a series of flavonoid, cinnamic acid and coumarin derivatives, it is suggested that the biological activity lays upon a common structural feature involving a phenolic ring bearing a side chain with conjugated double bonds and an oxygenated function, as found in the coumaroyl residue. These observations suggest that quercetin and related compounds may lead to a shift in intracellular protein phosphorylations by selectively inhibiting a particular type of protein kinase activity (CKG). It remains to be established whether this process may contribute to the mechanism of action of flavonoids upon cellular metabolism, particularly in the case of malignant cells.

Bioflavonoids such as quercetin inhibit the proliferation of different malignant cells *in vitro* [1]. This potentially useful antineoplastic activity is not yet fully understood with regard to its mechanism of action at the cellular level [2]. Whereas inhibition of various ATPase activities has been proposed to explain the effect of quercetin upon aerobic glycolysis in malignant cells [1, 3], this flavonoid was found able to increase cyclic AMP level and to impair DNA, RNA and protein synthesis in Ehrlich ascites tumor cells [4]. It has thus been assumed that quercetin may express its effects by acting on several different enzymatic systems. On the other hand, protein phosphorylations have appeared in the last years as a widely distributed cellular process in the regulation of several important metabolic pathways [5, 6]. Several protein kinase activities have been characterized and suggested to be involved in normal cellular proliferative cycle [7] as well as in proliferation of malignant cells [5, 8]. The effect of quercetin on a cyclic AMP-independent protein kinase has been reported in abstract form [9].

We have previously characterized in bovine adrenal cortex several cAMP-independent casein kinases, which could be classified into two major classes: the A type (CKA) using only ATP as phosphoryl donor and the G type (CKG) using GTP as well as ATP [10, 11]. It appeared of interest to examine the possible effect of flavonoid structures on these purified protein kinases. In addition to the possible definition of a new intracellular target site, which could contribute to the understanding of the cellular mechanisms of action of flavonoids, it was thought of interest to extend the study of this type of compounds to additional biological systems using nucleoside triphosphate as substrate.

This report describes the selective inhibitory effect of quercetin and related polyphenols on one of the cAMP independent casein kinases (CKG) isolated from bovine adrenocortical tissue, whereas CKA and cAMP-dependent protein kinase were not affected. The mechanism of this inhibitory effect was examined, and a structure–activity relationship was disclosed when considering the CKG inhibitory activities of a number of flavonoid and related structures. In addition to the selective inhibition of a particular type of protein kinase, which may be involved in the intracellular mechanism of action of flavonoids, these data show that quercetin may be used as an interesting tool in the biochemical characterization of the different types of protein kinases in cellular extracts.

MATERIALS AND METHODS

[γ^{32}P]ATP (20 Ci/mmole) and [γ^{32}P]GTP (25 Ci/mmole) were purchased from the Radiochemical Centre (Amersham, U.K.). Casein (Merck, Darmstadt, West Germany) was treated according to [12] before use. *p*-Hydroxybenzoic acid, *p*-coumaric acid, aesculetin, flavone, chrysin, kaempferol, quercetin, fisetin, taxifolin (dihydroquercetin), naringenin and rutin were obtained from Roth Laboratories (Karlsruhe, W. Germany). Ferulic and caffeic acids were from Fluka (Buchs, Switzerland). Cinnamic acid and coumarin were purchased from Prolabo (Paris, France). Histone (IIA) and rotenone were from Sigma Chemicals (St. Louis, U.S.A.). The structures of the major compounds tested as potential effectors of CKG activity are illustrated in Fig. 1.

Enzymatic preparations. Casein kinases of the A (CKA) and G (CKG) type were isolated from bovine

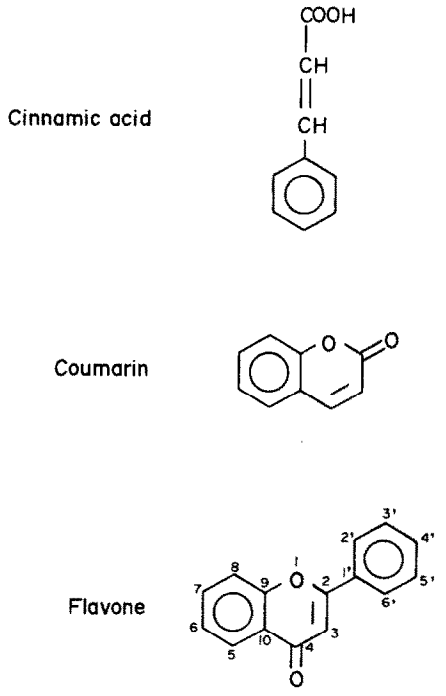

Fig. 1. Basic structures of the polyphenols and related compounds used in this work. *Cinnamic acid series*: cinnamic acid; coumaric acid (*p*-hydroxy-cinnamic acid); caffeic acid (*m*,*p*-dihydroxy-cinnamic acid); ferulic acid (*m*-methoxy, *p*-hydroxy-cinnamic acid). *Coumarin series*: coumarin; aesculetin (5,6-dihydroxy-coumarin). *Flavonoid series*: flavone; chrysin (5,7-dihydroxy-flavone); kaempferol (3,4',5,7-tetrahydroxy flavone); quercetin (3,3',4'5,7-pentahydroxy flavone); fisetin (3,3',4',7-tetrahydroxy-flavone); naringenin (4',5,7-trihydroxy-dihydro-flavone); taxifolin (3,3',4'5,7-pentahydroxydihydro-flavone); rutin (quercetin-3-rutinoside).

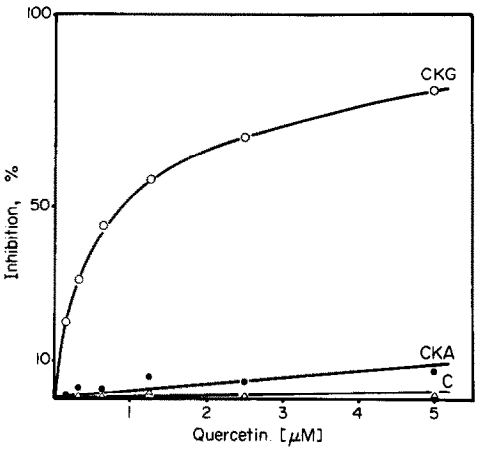

Fig. 2. Effect of quercetin on 3 different protein kinases. Catalytic subunit of the cAMP-dependent protein kinase (5 units: C), A type casein kinase (7.4 units: CKA) and G type casein kinase (5.4 units: CKG) activities were assayed in the presence of different concentrations of quercetin. Incubations were carried out with [γ^{32}P]ATP (10^{-5} M) and either histone (C) or casein (CKA and CKG) as the substrate. Mg^{2+} concentration in the medium was 5 mM for C and 50 mM for CKA and CKG assays. Results are expressed as per cent of inhibition with regard to the control.

adrenal cortex cytosol after differential adsorption onto a phosphocellulose column and a stepwise elution procedure as previously described [13]. The enzyme preparations were stored at −20° in 10 mM, pH 7.5 Tris–HCl buffer containing 1 mM dithiothreitol, 2% glycerol and 1 mg/ml bovine serum albumin (TDG buffer). Purified catalytic subunit of bovine heart cAMP dependent protein kinase was a kind gift from Professor J. G. Demaille.

Protein kinase assay. This was performed under standard assay conditions, using a trichloracetic acid precipitation procedure, as previously described [10] either with casein (at 50 mM $MgCl_2$ final concn) or histone (at 5 mM $MgCl_2$) as protein substrates. Various amounts of the individual compound to be tested were introduced in the assay as indicated in Results. Casein and histone kinase activity unit was defined as the amount of enzyme which catalyzed the incorporation of 1 pmole ^{32}P/min in the protein substrates under standard assay conditions.

All ancillary methods were as previously described [10, 11, 13].

RESULTS

Selective inhibition of G type casein kinase by quercetin

Figure 2 illustrates the effect of the presence of increasing concentrations of quercetin on the activities of three different isolated protein kinase catalytic subunit (C), A type (CKA) and G type (CKG) casein kinases. Whereas C and CKA activities were not significantly affected by quercetin concentrations in the micromolar range, CKG was significantly inhibited by quercetin at concentrations as low as 100 nM; 3 μM quercetin concentration brought about an average 80% inhibition of the enzyme. The quercetin concentration required to yield 50% inhibition of CKG (ID_{50}) under these conditions was 0.85 μM. When GTP was used as phosphoryl donor instead of ATP, similar inhibitory effect of quercetin was observed upon CKG activity (Table 1), whereas in this case, C and CKA could not be assayed since these enzymes are not able to use this nucleotide as a source of phosphate [10, 11].

Table 1. Effect of ATP and GTP concentrations on CKG inhibition by quercetin

Nucleotide concn (μM)	% Inhibition of CKG ATP as substrate	GTP as substrate
1	84	68
5	79	65
10	73	61
50	50	32
100	30	14

G-type casein kinase (5.3 units) was incubated in the presence of 2.5 μM quercetin with different concentrations of ATP or GTP. Results are expressed as per cent of CKG inhibition with regard to the control in the absence of quercetin.

Mechanism of CKG inhibition by quercetin

The effect of quercetin (2.5 μM) on CKG activity was examined at different concentrations of phosphoryl donor (ATP or GTP). As seen in Table 1, the inhibitory effect of the flavonoid appeared dependent upon the nucleotide concentration in both cases. These data confirmed that quercetin inhibits CKG activity regardless of the nature of the phosphate donor and suggested that the flavonoid may affect the enzyme through an impairment of the enzyme–nucleotide interaction. In addition, it was observed that varying CKG protein substrate (casein) concentration in the assay did not influence the inhibitory effect of quercetin (not shown).

In order to further characterize the mechanism of CKG inhibition by quercetin, the enzyme activity was assayed at various ATP concentrations and in the presence of increasing quercetin concentrations. The corresponding data could be plotted using a Lineweaver–Burk graph, as shown in Fig. 3, and indicated that quercetin behaves as a competitive inhibitor toward ATP in the phosphorylation reaction. Using these data, an inhibitory constant K_i of 0.75 μM could be calculated for quercetin. The same experimental approach indicated that quercetin was similarly a competitive inhibitor of GTP and a corresponding K_i of 0.22 μM was calculated. Since the K_m of CKG for ATP has been previously determined to be about 7 μM [10, 11], it may be inferred that quercetin exhibits an affinity for CKG which is about one order of magnitude higher than that of ATP.

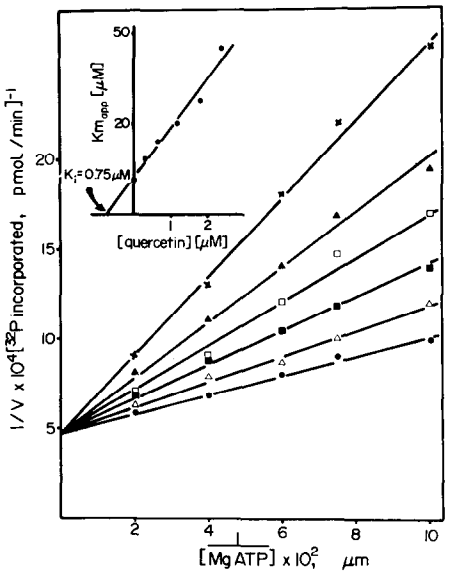

Fig. 3. Lineweaver–Burk plots for CKG activity with ATP as substrate in the presence of various concentrations of quercetin. Conditions were 10 mM Tris–HCl pH 7.5, 50 mM $MgCl_2$, casein (600 μg) and quercetin: 0 (●——●); 10 μM (△——△); 12.5 μM (■——■); 16.7 μM (□—□); 25 μM (▲—▲) or 50 μM (×—×). Assays were initiated by the addition of 5.4 units of CKG. Incubation time was 5 min. Each point is the mean of three replicates. Inset: plot of the slopes of the various reciprocal graphs vs corresponding quercetin concentrations.

Effect of various flavonoids and related structures on CKG activity

In order to examine whether the inhibitory effect of quercetin upon CKG activity was a general property of flavonoid structures, a number of individual compounds were tested under the aforementioned assay conditions. In addition, with the aim of gaining some information concerning a structure–activity relationship, some structurally related compounds such as cinnamic acids and coumarins (Fig. 1) were

Table 2. Inhibition of G-type casein kinase activity by various flavonoid structures and related compounds*

Compound added	Protein kinase activity (pmoles ^{32}P incorporated/min)	Inhibition (%)
None	3.15	0
p-Hydroxy-benzoic acid	3.06	3 ± 1.7
Cinnamic acid	2.7	15 ± 2.3
Coumaric acid	2.3	26 ± 2.4
Caffeic acid	2.2	29 ± 0.4
Ferulic acid	1.9	39 ± 3.1
Coumarin	3.03	3.8 ± 0.8
Aesculetin	3.02	4.0 ± 1.3
Flavone	2.90	7.9 ± 2.3
Rotenone	2.90	8.2 ± 0.8
Chrysin	1.90	39.6 ± 1.7
Kaempferol	1.60	49.2 ± 1.3
Quercetin	1.20	61.9 ± 2.2
Fisetin	1.00	68.2 ± 1.3
Rutin	2.20	11.0 ± 0.5
Taxifolin	2.83	9.0 ± 1.9
Naringenin	2.99	5.0 ± 2.3

* The incubation conditions were as described in Materials and Methods, in a final 80 μl vol. containing casein (600 μg), [γ^{32}P]GTP (10^{-5} M), 50 mM $MgCl_2$ and 2.5 μM of various flavonoids or related compounds in TDG buffer. The reaction was initiated by addition of 3 units of purified casein kinase. Each point is the mean of 3 independent replicates ± S.E.M.

included in these studies. The corresponding data are collected in Table 2 and showed that inhibition of CKG was not a property limited to quercetin. In fact fisetin, which is devoid of one hydroxyl group of quercetin at C_5 was even more potent than quercetin itself. The polycyclic structure of the flavonoid series does not appear to be a prerequisite for the biological activity since cinnamic acid-like compounds exhibited a significant CKG inhibitory activity. On the other hand, representative structures (i.e., *p*-hydroxybenzoic acid, ferulic acid, coumarin, chrysin and fisetin) were examined with regard to their effect on casein kinase A and cAMP-dependent protein kinase. All of these compounds showed negligible effect on these two protein kinase activities, suggesting that the selective inhibition exhibited by quercetin toward the G type casein kinase was a common feature of all the potent structures tested.

DISCUSSION

In addition to the previously reported effects of quercetin on several cellular enzymatic processes including ATPase activities, macromolecular biosynthesis, cAMP metabolism [1–4], this report describes a potent inhibitory action of the flavonoid upon a particular protein phosphorylation system. Three different purified protein kinases were used in this study; whereas cAMP-dependent protein kinase (purified catalytic subunit) and A type (using only ATP) casein kinase activities were not influenced by quercetin, the flavonoid selectively inhibits the G type casein kinase with either ATP or GTP as phosphate donor. Study of the mechanism of inhibition indicated that quercetin acts as a competitive inhibitor toward the nucleoside triphosphate ATP and/or GTP. These observations show that quercetin is not a general competitor of ATP at the protein kinase level and point to a specific interaction between G type casein kinase and the flavonoid at the nucleotide site which in this case can accept both ATP and GTP [10, 11]. Although the ability of quercetin to form a complex with ATP has been suggested to possibly explain the inhibition of ATPase activities [14], the selective inhibition of G type casein kinase does not support such a general mechanism of action of the drug.

Consideration of the structure–activity relationship (Table 2) shows that fisetin is an even better inhibitor of CKG than quercetin. The series of structures tested in this study suggests that the C_2–C_3 insaturation of the heteroatomic ring of the flavonoid nucleus is required for the CKG inhibitory potency since naringenin and dihydroquercetin (taxifolin) exhibit negligible inhibitory effect. Polar substituents (hydroxyl groups) on the adjacent fused ring appear to greatly increase the biological activity of the corresponding flavonoids (see the effect of flavone vs that of chrysin). Hydroxylation of the phenyl ring also leads to substantial enhancement of the inhibitory property of the flavonoid (e.g., chrysin versus quercetin or fisetin). These structural requirements are partly found in the cinnamic acid related structures which exhibit a potent inhibitory effect on CKG activity. It may thus be suggested that the biological activity lays on a structure represented by a *meta*, *para*, dihydroxylated aromatic C_6 ring, bearing a side chain with conjugated unsaturations and an oxygenated substituent (ketone or carboxyl). Methylation of a phenolic group (e.g., in ferulic acid) may even slightly increase the biological potency. On the other hand, introduction of a bulky substituent such as rutinose with an osidic linkage at C-3 greatly impairs the CKG inhibitory property (e.g., rutin).

These structural requirements may suggest a rather polar site for the active compounds at the enzyme level, a character which is compatible with a competitive interaction of ATP (or GTP) at the same site.

These observations are of interest in view of the possibility of developing molecular probes useful as mapping agents in the study of active site of protein kinases, and especially G type casein kinase, using affinity labeling techniques. These structures may be also useful as ligand for G type casein kinase in affinity chromatography techniques as applied to the purification of this type of enzyme in cellular extracts.

The biological significance of the reported observations remains to be established. However, since quercetin has been reported to increase cyclic AMP concentration in ascites cells [15], this should lead to the intracellular increase of cAMP-dependent protein kinase activity. At the same time, G type casein kinase activity will be inhibited by quercetin. This would result in a drastic unbalance in intracellular phosphorylations since cAMP-dependent and G type protein kinases have each a specific set of protein substrates. It remains to be demonstrated whether this phenomenon may be involved in the mechanism of action of flavonoids on cellular metabolism [1–4]. On the other hand, due to their selective G type casein kinase inhibition, quercetin, fisetin and related structures appear as interesting probes for the biochemical characterization of protein kinases in tissue extracts and for the study of the role of protein phosphorylation systems in the regulation of cellular functions.

Acknowledgements—This work was possible thanks to the financial support of the INSERM (U-244; ATP 77-84 and 79-114) the CNRS (ERA 942) and the Fondation pour la Recherche Médicale Française. We are indebted to Dr Tissut for helpful discussions throughout this work.

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