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PROTEIN KINASE C INHIBITION BY PLANT FLAVONOIDS

KINETIC MECHANISMS AND STRUCTURE–ACTIVITY RELATIONSHIPS*

PATRICE C. FERRIOLA,†‡ VIVIAN CODY§ and ELLIOTT MIDDLETON, JR.†‖

†Department of Medicine, State University of New York at Buffalo, Buffalo General Hospital, Buffalo, NY 14203; and §Department of Molecular Biophysics, Medical Foundation of Buffalo, Inc., Buffalo, NY 14203, U.S.A.

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Abstract—Protein kinase C (PKC) from rat brain was inhibited by plant flavonoids in a concentration-dependent manner depending on flavonoid structure. Of the fifteen flavonoids studied, fisetin, quercetin and luteolin were the most potent, while hesperetin, taxifolin and rutin were among the least potent. The flavonol fisetin was almost 100% inhibitory at a concentration of 100 μM. The extent of inhibition was the same whether diacylglycerol or 12-*O*-tetradecanoylphorbol-13-acetate was used as enzyme activator. Inhibition was independent of Ca^{2+}, phospholipid, and enzyme activator, as shown by inhibition of protamine phosphorylation in the absence of the regulatory components. Fisetin was a competitive inhibitor with respect to ATP binding and noncompetitive with respect to protein substrate. The X-ray crystal structure analysis of hesperetin monohydrate showed that the molecule is essentially planar despite the sofa conformation of the γ-pyran ring and the 27° twist of the 2-phenyl ring. Comparison of this inactive flavanone with those of the active flavones showed that, although hesperetin can adopt a planar profile similar to those of fisetin and quercetin, the 4′-methoxy substituent blocks an essential structural feature required for inhibitory activity. Analysis of these structure–activity data revealed a model of the minimal essential features required for PKC inhibition by flavonoids: a coplanar flavone structure with free hydroxyl substituents at the 3′, 4′ and 7-positions.

Flavonoids are phenolic compounds synthesized by vascular plants, fruits, and vegetables which have varied effects on mammalian cell systems [for review see Refs. 1 and 2]. *In vivo*, the flavonoid quercetin inhibits tumor promotion by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and teleocidin in the two-stage model of carcinogenesis in mouse epidermis [3, 4]. At the cellular level, flavonoids from different chemical classes inhibit TPA-, teleocidin- and aplysiatoxin-induced histamine release from human basophils [5, 6]. These effects may be mediated through inhibition of protein kinase C (PKC) since PKC is the cellular receptor for these tumor promoters [7, 8]. Flavonoids have been reported to inhibit partially purified PKC activated by TPA [9], and teleocidin [10]; however, the mechanism of this inhibition has not been identified, nor have the flavonoids been shown to inhibit PKC when unsaturated diacylglycerol (DAG), an endogenous PKC activator, is used to stimulate the enzyme. Further study of flavonoid inhibition of PKC is warranted since flavonoid PKC inhibitors may prove useful agents for modulating PKC activity *in vitro* and *in vivo*. In addition, the ubiquitous distribution of PKC in mammalian cells suggests that naturally occurring flavonoids could exert effects on many cell types and biochemical reactions through inhibition of the enzyme.

This study was designed to characterize the effect of selected flavonoid compounds on PKC isolated from rat brain and to determine the kinetic mechanism of PKC inhibition. We found certain flavonoids, depending on structure, to be potent inhibitors of both TPA- and DAG-activated PKC. To explore further the structure–activity relationships, the X-ray crystal structure analysis of hesperetin was carried out and compared with those of the potent PKC inhibitors.

MATERIALS AND METHODS

Chemicals. Histone-IIIS, phosphatidylserine (PS), diolein, TPA, leupeptin, phenylmethylsulfonyl fluoride (PMSF), DEAE cellulose, hesperetin, phloretin, naringin, (+)-catechin and taxifolin were obtained from the Sigma Chemical Co., St. Louis, MO; fisetin, quercetin, rutin and chalcone were from the Aldrich Chemical Co., Milwaukee, WI; formononetin, luteolin, isorhamnetin and apigenin were from Sarget Laboratories, Merignac, France; and nobiletin and tangeretin were provided by Mr. James Tatum, U.S. Department of Agriculture, Lakeland, FL. Nitrocellulose filters (0.45 μm pore size) were obtained from the Millipore Corp., Bedford, MA; [γ-^{32}P]ATP was from ICN Radiochemicals, Irvine, CA. Male Sprague–Dawley rats were purchased from Blue Spruce Farms, Altamont, NY.

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‡ Present address: National Institute of Environmental Health Science, Laboratory of Pulmonary Pathobiology, Research Triangle Park, NC 27709.

‖ Correspondence: Dr. Elliott Middleton, Jr., Department of Medicine, SUNY at Buffalo, Buffalo General Hospital, 100 High St., Buffalo, NY 14203.

Purification of rat brain PKC. PKC was partially purified from rat brain by DEAE cellulose column chromatography in a procedure slightly modified from that of Kikkawa *et al.* [11]. Male Sprague–Dawley rats were decapitated, and brains were removed and dissected from the brain stem and cerebellum. Cerebral cortexes were placed in a homogenization buffer at 0–4° which contained 20 mM Tris, pH 7.5, 10 mM ethyleneglycolbis-(aminoethylether)tetra-acetate (EGTA), 2 mM EDTA, 250 mM sucrose, 0.2 mg/ml PMSF and 0.1 mg/ml leupeptin. Five to ten rats (175–200 g each) were used for each purification. After homogenization and centrifugation at 100,000 *g* for 60 min, the supernatant fraction was applied to a DEAE cellulose anion exchange column (12 cm × 1.5 cm) previously equilibrated with 20 mM Tris, pH 7.5, containing 10 mM 2-mercaptoethanol, 1 mM EDTA and 1 mM EDTA. The enzyme was eluted with 250 ml of a 0–300 mM linear salt gradient in the equilibration buffer.

The final preparations showed a 10- to 30-fold purification over the crude supernatant fractions. Eadie–Scatchard plots (velocity/[substrate] versus velocity) yielded straight lines, indicating the presence of a single enzyme catalyzing the histone-IIIS phosphorylation [12]. The specific activity of the enzyme was 6.9 ± 1.8 nmol · min^{-1} · (mg protein)$^{-1}$ for twelve assays. Protein concentration was determined by the method of Bradford [13] using bovine serum albumin as standard and was 1.9 ± 0.5 mg/ml for twelve assays.

PKC assay. PKC activity was determined at 30° in a 0.2-ml volume reaction mixture containing 25 mM Tris, pH 7.5, 0.6 mM $CaCl_2$, 1.25 mM $MgCl_2$, 0.2 mg/ml histone-IIIS, 0.04 mg/ml PS, 2–10 μg/ml protein and either 0.8 μg/ml diolein (as the diacylglycerol) or 10 ng/ml TPA. PS and diolein were prepared as described by Kikkawa *et al.* [11]. The reaction began with the addition of [γ-^{32}P]ATP (1–4 nmol, 1–5 × 10^5 cpm/nmol). Unless otherwise stated, flavonoids were added immediately prior to ATP addition. All flavonoids were dissolved in Me_2SO and prepared as fresh stock solutions for each assay. Final Me_2SO concentrations in the assay mixture did not exceed 0.25%. The same concentration of Me_2SO was added to the control reaction. Background activity was determined by omission of Ca^{2+}, PS and activator (either diolein or TPA) from the reaction mixture and was subtracted from PKC activities. After 3 min the reaction was stopped by the addition of 3 ml of cold 25% trichloroacetic acid (TCA) and by filtration over nitrocellulose filters. Filters were washed three times with cold 10% trichloroacetic acid. Radioactivity was determined by liquid scintillation counting. PKC activity is expressed as nanomoles of phosphate incorporated into histone-IIIS per minute per milligram of protein.

PKC activity was also determined by an alternative assay using protamine rather than histone-IIIS as protein substrate and carried out in the absence of Ca^{2+}, PS and activator [14–16]. Reaction mixtures contained 25 mM Tris, pH 7.5, 6.25 mM $MgCl_2$ and 0.5 mg/ml protamine. Background activity was determined by replacing protamine with histone-IIIS. The reaction was terminated by addition of cold 10% TCA and filtration as described above.

Crystallography. Crystals of hesperetin monohydrate (Sigma) were grown at room temperature from an aqueous solution and are triclinic, space group P-1 with lattice parameters a = 6.841(2), b = 9.082(2), c = 12.170(3) Å, $\alpha = 72.85(2)$, $\beta = 84.32(2)$, $\gamma = 81.16(2)°$, and z = 2. Precise lattice parameters were calculated by least-squares analysis of 25 reflections with a 2θ range 20.15 to 30.08° for a crystal with dimensions 0.2 × 0.24 × 0.44 mm. The intensities of 4192 independent reflections [$3000 > 3\sigma(I)$] were collected with MoKα radiation on a Nicolet P3 automated diffractometer with an Nb filter. Corrections were made for Lorentz and polarization effects, but not for absorption or extinction effects. The structure was solved by the use of the direct methods programs MULTAN [17] and NQEST [18] and was refined by full-matrix anisotropic least-squares techniques. The function $\Sigma w(|F_o| - |F_c|)^2$ was minimized, where w is based on diffractometer counting statistics. The final residual, $R = \Sigma(|F_o| - |F_c|)/|F_o|$, was 0.079 for 3000 data.

Table 1. Hesperetin hydrate atomic coordinates (X10**4) and equivalent isotropic thermal parameters (X10**2)

Atom	X/A(σ)	Y/B(σ)	Z/C(σ)	BEQ(σ)
C(2)	6086(4)	2454(3)	−382(2)	304(7)
C(3)	6666(4)	3698(3)	−1435(2)	316(7)
C(4)	6037(3)	3451(3)	−2511(2)	281(6)
C(4A)	4367(3)	2605(3)	−2372(2)	257(6)
C(5)	3607(3)	2329(3)	−3322(2)	283(6)
C(6)	1959(4)	1594(3)	−3204(2)	290(6)
C(7)	989(3)	1155(3)	−2127(2)	279(6)
C(8)	1664(3)	1416(3)	−1160(2)	295(7)
C(8A)	3360(3)	2102(3)	−1289(2)	254(6)
C(1′)	6521(3)	2705(3)	743(2)	291(7)
C(2′)	5370(4)	2154(3)	1741(2)	294(7)
C(3′)	5826(3)	2309(3)	2778(2)	285(6)
C(4′)	7474(3)	3010(3)	2837(2)	284(6)
C(5′)	8622(4)	3556(4)	1852(2)	396(8)
C(6′)	8143(4)	3410(4)	810(2)	421(9)
O(1)	4005(2)	2324(2)	−331(1)	322(5)
O(4)	6892(3)	3978(2)	−3454(1)	427(6)
O(5)	4504(3)	2835(2)	−4381(1)	404(6)
O(7)	−666(3)	453(2)	−1955(2)	388(6)
O(3′)	4660(3)	1737(3)	3738(2)	484(7)
O(4′)	7799(3)	3093(2)	3911(1)	352(5)
C(4′1)	9399(4)	3898(4)	3989(2)	362(8)
O(W1)	1644(4)	−86(3)	3972(2)	503(8)
H(2)	688(4)	140(3)	−47(2)	41(6)
H(3A)	807(4)	375(3)	−149(2)	34(6)
H(3B)	586(5)	471(4)	−133(3)	62(8)
H(6)	150(3)	142(3)	−386(2)	27(5)
H(8)	92(4)	113(3)	−40(2)	36(6)
H(2′)	423(4)	168(3)	170(2)	39(6)
H(5′)	981(4)	404(3)	190(2)	46(7)
H(6′)	909(4)	379(3)	16(3)	43(6)
H(51)	555(5)	334(4)	−431(3)	59(8)
H(71)	−94(5)	35(4)	−258(3)	58(9)
H(3′1)	484(6)	209(4)	427(4)	71(10)
H(4′A)	1063(4)	339(3)	378(2)	31(6)
H(4′B)	924(4)	383(3)	482(2)	43(6)
H(4′C)	931(5)	500(4)	347(3)	55(8)
H(WA)	145(7)	−72(6)	459(4)	95(15)
H(WB)	257(5)	35(4)	389(3)	55(9)

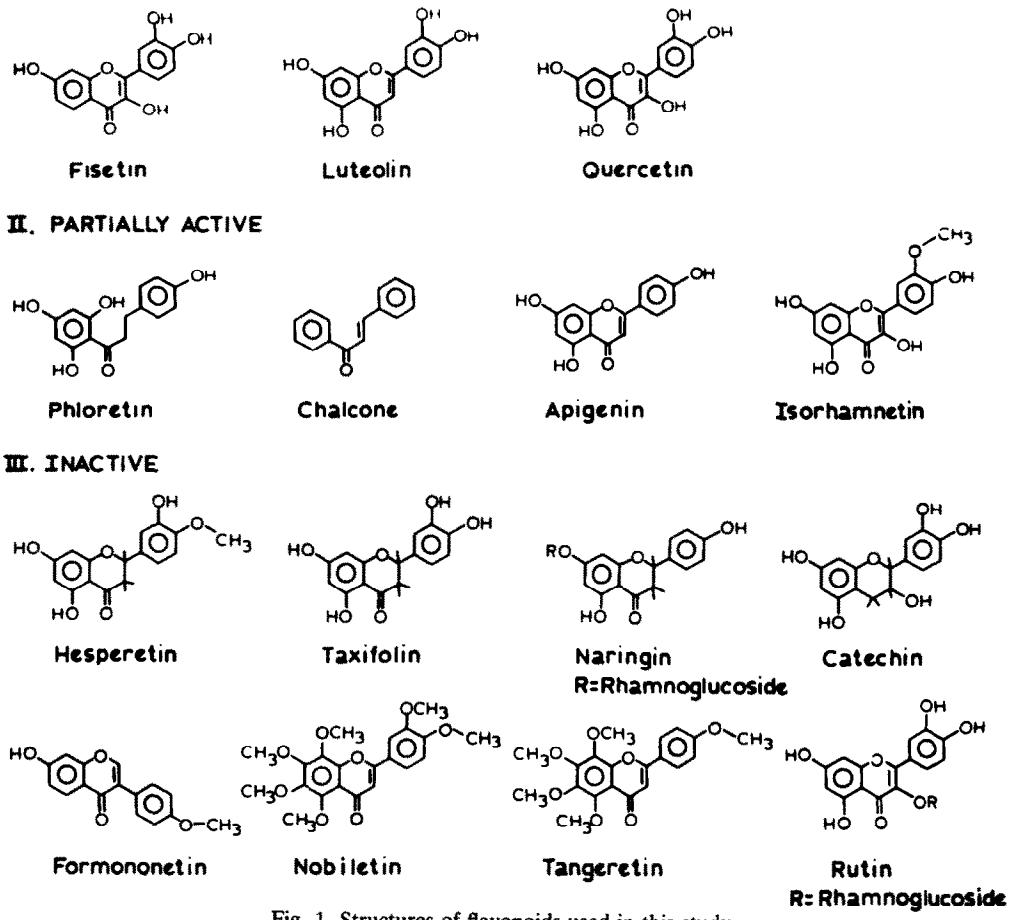

Fig. 1. Structures of flavonoids used in this study.

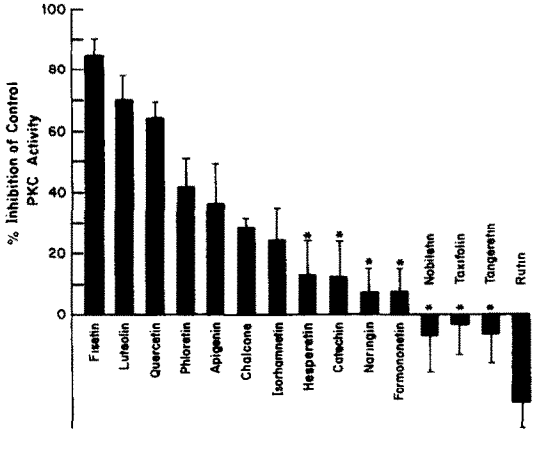

Fig. 2. Effect of flavonoid compounds on PKC activity. The effects of 50 μM concentrations of fifteen different flavonoids on control PKC activity were determined. Assays were carried out as described in Materials and Methods using 5 μM ATP and 10 μg protein. Diolein was used as enzyme activator. Values represent the mean percent inhibition of control PKC ± the SEM of at least three independent experiments using three different enzyme preparations. Maximal PKC activity (100%) was equal to 9.7 ± 4.6 nmol · min^{-1} · (mg protein^{-1}). Key: (*) not significantly different from zero.

Atomic scattering factors are from *International Tables for X-ray Crystallography* [19]. Hydrogen atoms were located in difference Fourier synthesis and were refined isotropically. The fractional coordinates and equivalent isotropic thermal parameters are listed in Table 1.

RESULTS

Inhibition of PKC by flavonoid compounds. Fifteen flavonoids from the chemical classes in Fig. 1 were used in the study. Seven of the compounds tested inhibited PKC activity in a concentration-dependent manner with varying potencies. The effects of 50 μM final concentrations of the flavonoids on PKC activity are shown in Fig. 2. Diolein was used as the PKC activator in these assays. The control PKC activity for nine separate enzyme preparations used for these assays was 9.7 ± 4.6 nmol · min^{-1} · (mg protein)$^{-1}$. Fisetin, luteolin and quercetin were the most potent flavonoids, inhibiting PKC activity by 85, 70 and 64%, respectively, at a concentration of 50 μM. The concentration–response relationship for fisetin inhibition of PKC activity is shown in Fig. 3. PKC activity was inhibited by 50% at a fisetin concentration between 10 and 25 μM and almost 100% at a 100 μM concentration.

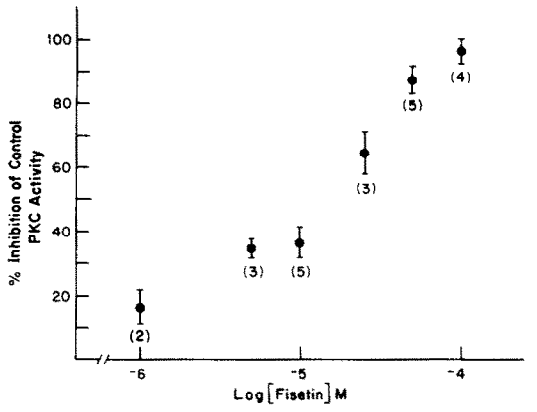

Fig. 3. Concentration–response relationship for fisetin inhibition of PKC activity. PKC activity was determined using diolein as activator, 5 μM ATP and 10 μg protein as described in Materials and Methods. Numbers in parentheses indicate the number of independent experiments using different enzyme preparations. Values represent means ± SEM. See legend of Fig. 2 for control PKC activity.

Four other compounds, i.e. phloretin, apigenin, chalcone and isorhamnetin, inhibited PKC activity less than 50% of the control activity at 50 μM. The remaining eight flavonoids did not inhibit PKC activity significantly. Rutin, the 3-rhamnosylglucoside of quercetin, significantly increased PKC activity by 19%. We did not explore this relationship further except to note that a mixture of 50 μM fisetin and 50 μM rutin resulted in the same degree of inhibition of PKC as 50 μM fisetin alone (data not shown).

The effect of flavonoid incubation time on PKC activity was examined using the following incubation protocols (Table 2). In the standard assay, enzyme and flavonoid were added simultaneously to a 4° reaction mixture containing Ca^{2+}, Mg^{2+}, PS and diolein. The tubes were then placed in a 30° water bath, they were temperature equilibrated for 4 min and the reaction was started with the addition of [γ-^{32}P]ATP. To study the effect of flavonoid incubation time on PKC activity, the control conditions were changed in the following ways. Fisetin was added to the reaction mixture: (a) with addition of the enzyme (standard), (b) 15 min prior to addition of enzyme instead of simultaneously, (c) 15 min after the addition of enzyme, and (d) with [γ-^{32}P]ATP. There was no statistically significant difference between the amount of fisetin inhibition under the standard conditions and under the alternative pre-assay conditions. In a representative experiment, control PKC activity was 23.4 nmol · (mg protein)$^{-1}$ · min^{-1} and under standard conditions fisetin inhibited PKC activity by 83%. Pre- and post-enzyme incubations yielded inhibition of 77 and 87%, respectively, and when the reaction began with the addition of enzyme, inhibition was 82%.

To determine whether the flavonoids were equally active or inactive using exogenous or endogenous activators of PKC, the effects of the compounds on PKC activity were assessed using either diolein or TPA to activate the enzyme. As shown in Fig. 4, a significant correlation exists between flavonoid inhibition of PKC when activated with diolein or when activated with TPA. The flavonoids inhibited PKC with a similar order and magnitude of potency when either endogenous or exogenous type PKC activators were used to stimulate the enzyme.

Flavonoid structure–activity relationships. The flavonols and flavones (Fig. 1) proved to be the most active inhibitors of PKC as demonstrated by fisetin, quercetin and luteolin. Elimination or methylation of the 3′-OH group reduced inhibitory potency within these chemical classes as shown by isorhamnetin and apigenin. Inhibitory potency of the flavonols and flavones was eliminated by glycosylation (e.g rutin) and by methylation of the ring OH groups (e.g. nobiletin and tangeretin). Saturation of the pyrone ring C2–C3 bond of flavonols and flavones yields flavanonols and flavanones, respectively. Flavonoids from these chemical classes were without activity as inhibitors of PKC, as demonstrated by taxifolin, the flavanonol of quercetin, and by the flavanones,

Table 2. Effect of flavonoid incubation time on PKC activity

Condition		PKC activity [nmol product formed · min^{-1} · (mg protein^{-1})]	% Inhibition
	Control	23.4	
(A)	Standard	4.0	83
(B)	Pre-enzyme	5.4	77
(C)	Post-enzyme	3.1	87
(D)	With ATP	4.2	82

Control values for PKC activity were determined using diolein and the reaction mixture described in Materials and Methods. Fisetin inhibition (50 μM concentration) of PKC was determined either by (A) adding the flavonoid simultaneously with the enzyme to the reaction mixture (standard assay), (B) adding the flavonoid to the reaction mixture 15 min prior to adding the enzyme (pre-enzyme), (C) adding the flavonoid to the reaction mixture 15 min after addition of enzyme (post-enzyme) or (D) adding the flavonoid and [γ^{32}P]ATP to the reaction mixture and beginning the reaction with addition of enzyme (with ATP). Results are shown for a single experiment. Similar results were observed with two different enzyme preparations.

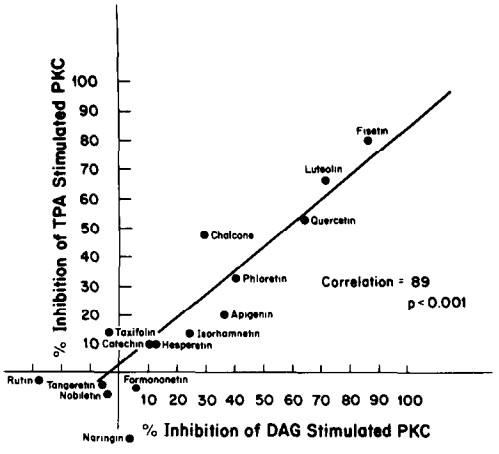

Fig. 4. Correlation of flavonoid inhibition of DAG- and TPA-activated PKC. The percent inhibition of PKC activity by flavonoids is shown using diolein or TPA as enzyme activator as described in Materials and Methods. Results are averages of at least two independent experiments using different enzyme preparations.

hesperetin and naringin. Naringin is glycosylated which also leads to inactivity. Neither the isoflavone, formononetin, nor the flavan, (+)-catechin, was an inhibitor of PKC activity. The open chain flavonoid congeners chalcone and phloretin were moderate inhibitors of PKC activity.

Mechanism of flavonoid inhibition of PKC. To determine the mechanism of flavonoid inhibition of PKC, protamine rather than histone-IIIS was used as the phosphate acceptor in certain assays of PKC activity. Protamine is phosphorylated by PKC in the absence of PS, Ca^{2+}, and activator, requiring only Mg^{2+} and ATP [14–16]. In a representative experiment (Table 3), PKC activity using histone-IIIS as substrate was 11.1 and 12.2 nmol · min^{-1} · (mg protein)$^{-1}$ using diolein and TPA as activators, respectively. The activity of PKC when stimulated with either diolein or TPA was maximal, i.e. no

Table 3. Control values for PKC activity using histone-IIIS or protamine as protein substrate

Substrate	Activator	PKC activity [nmol product formed · min^{-1} · (mg protein)$^{-1}$]
Histone	None	4.2
Histone	DAG	11.1
Histone	TPA	12.2
Protein	None	10.8

Assays with histone-IIIS as protein substrate required Ca^{2+}, PS and activator as described in Materials and Methods. Histone-IIIS (0.25 mg/ml), DAG (1.0 μg/ml) and TPA (0.01 μg/ml) were used for these assays. Assays using protamine as substrate required only Mg^{2+} and used 0.5 mg/ml of protamine. Reactions began with addition of 5 μM ATP. The following are results from a single experiment using the same enzyme preparation. Equal amounts of protein were used for each condition.

Table 4. Effects of flavonoids on PKC phosphorylation of protamine

Additions	PKC activity [nmol product formed · min^{-1} · (mg protein)$^{-1}$]	% Inhibition
Control	10.4 ± 1.3	
Rutin	11.7 ± 1.8	0
Quercetin	4.1 ± 1.0*	61
Fisetin	1.7 ± 1.0*	84

Assays using protamine as protein substrate were performed as described in Materials and Methods. All flavonoids were present at 50 μM concentrations. Protamine concentration was 0.5 mg/ml. Results are means ± SEM of at least three independent experiments.

* Statistically different from control, $P < 0.01$ (Student's *t*-test).

greater PKC activity was seen with increased PS, diolein or TPA at the same enzyme and histone concentration. The activity of PKC when 0.5 mg/ml of protamine was used as substrate was 10.8 nmol · min^{-1} · (mg protein)$^{-1}$ which was the same activity as when maximally stimulated. The effects of three flavonoids on PKC activity using protamine as substrate are shown in Table 4. Both quercetin and fisetin inhibited PKC activity, whereas rutin had no effect.

The results of kinetic analysis of flavonoid inhibition of PKC are shown in Fig. 5. The effect of increasing concentrations of fisetin while the ATP concentration was varied (Fig. 5A) yielded double-reciprocal plots which indicate competitive inhibition. Double-reciprocal plots of increasing fisetin concentration and varied histone concentrations indicate noncompetitive inhibition (Fig. 5B). Inhibition was also noncompetitive when the protamine concentration was varied (data not shown). The K_m for ATP and histone averaged 8.3 ± 0.5 μM and 10.0 ± 2.0 μg/ml for four and three independent experiments, respectively. The K_i averaged 4.6 ± 0.2 μM for ATP and 2.5 ± 0.1 μg/ml for histone. The IC_{50} for fisetin when determined using an ATP concentration of 5 μM was 10 μM. Luteolin inhibition of PKC also displayed competitive kinetics with respect to ATP and noncompetitive kinetics with respect to protein substrate (data not shown).

Crystallography. To understand the mechanism of PKC inhibition by the flavonoids, their molecular conformations, determined crystallographically, were compared using computer graphics techniques. The crystal structures of quercetin and phlorizin have been described previously [20, 21].

The molecular structure of hesperetin (Fig. 6) shows that the γ-pyran ring is in a sofa conformation, as observed in several other flavanone structures [20–22]. Although the phenyl ring was twisted 27° (01-C2-C1′-C2′) from the benzopyrone ring system, the overall conformation was flat. This twist value was at the low end of the range (26–104°) observed for flavonoid structures [22]. There was an intramolecular hydrogen bond between the 05 hydroxyl and 04 ketone and also an extended network of intermolecular hydrogen bonds among the 07 and

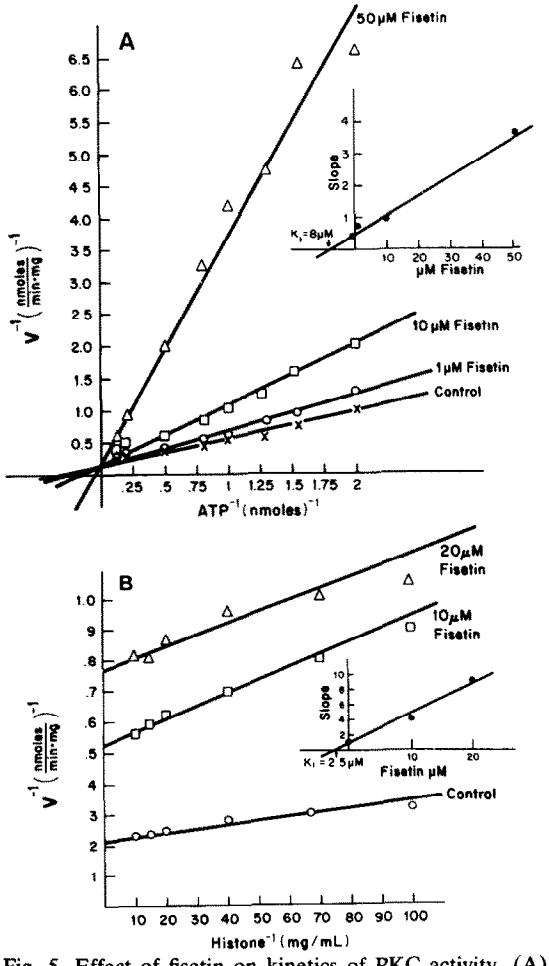

Fig. 5. Effect of fisetin on kinetics of PKC activity. (A) Effect of varied ATP concentration. PKC (10 μg protein) was incubated with different concentrations of fisetin (0, 1, 10 and 50 μM) for 4 min prior to addition of [γ-^{32}P]ATP. The assay was carried out as described in Materials and Methods. Data for a single experiment are represented graphically by the double-reciprocal method. K_i was determined graphically from the replot of the slope of the double-reciprocal plot and the fisetin concentration as shown in the insert. Similar results were obtained for four independent experiments. (B) Effect of varied protein substrate concentration. In this experiment, 0, 10 or 20 μM fisetin was incubated with PKC and the histone-IIIS concentration varied at a constant ATP concentration of 5 μM (1 nmol). Similar results were obtained for two independent experiments using histone as substrate and two experiments using protamine as substrate.

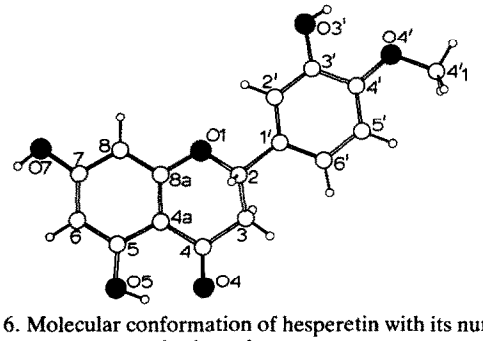

Fig. 6. Molecular conformation of hesperetin with its numbering scheme.

03′ hydroxyl groups and the water molecule in the crystal. A similar hydrogen bonding pattern has been observed in the crystal structure of quercetin hydrate [20].

DISCUSSION

Protein kinase C is an important effector of transmembrane signals invoked by the polyphosphoinositide pathway [23]. This pathway is characterized by a cycle of membrane phosphoinositide turnover initiated by ligand–receptor interactions at the cell membrane. Ligands initiating phosphoinositide turnover include hormones, neurotransmittors and growth factors. DAG and inositol trisphosphate are products of phosphoinositide turnover which serve as second messengers in converting the membrane signal to an intracellular response. DAG directly activates PKC, whereas inositol trisphosphate mobilizes intracellular Ca^{2+} and indirectly contributes to PKC activation. When activated, PKC phosphorylates the serine and threonine residues of protein substrates. The uniquitous tissue distribution of PKC indicates that it plays a role in the regulation of many enzymes and other proteins. It follows, therefore, that inhibition of PKC would have important physiological consequences. In this study we demonstrate that PKC was inhibited by flavonoids in a concentration-dependent manner depending on flavonoid structure. The mechanism of inhibition was independent of the type of enzyme activator used and was also independent of PKC regulatory components.

The mechanism of flavonoid inhibition of PKC is not known. A major goal of this study was to determine the kinetics of inhibition and to analyze flavonoid structure–activity relationships. Flavonoids could directly affect PKC activity by interfering with enzyme activation or phosphorylation. Transfer of the terminal phosphate of ATP to a protein substrate first requires activation of PKC by Ca^{2+}, phospholipid and DAG (or tumor promoter). The primary structure of PKC includes a hydrophobic regulatory domain which contains the binding sites for phospholipid, Ca^{2+} and DAG or phorbol esters, and a hydrophilic catalytic domain which contains an ATP and protein substrate binding site. Therefore, flavonoids could interfere with the binding of one or more components to the regulatory and/or catalytic domains. Our data suggest that flavonoids *do not* interact with the regulatory domain since PKC phosphorylation of protamine was inhibited by flavonoids in the absence of the regulatory components Ca^{2+}, PS and DAG. The concentration dependency and order of potency were similar for flavonoid inhibition of both protamine and histone-IIIS phosphorylation by PKC. The data also showed, for the first time, that flavonoids are equally effective PKC inhibitors whether DAG or TPA is used as an enzyme activator. These data are important to note since DAG is the endogenous cellular activator of PKC, whereas TPA is an exogenous activator and may have effects on cell function not related to PKC activity [23]. Since flavonoid inhibition of PKC activity was independent of regulatory components, we examined whether the compounds interfered with components of the catalytic subunit.

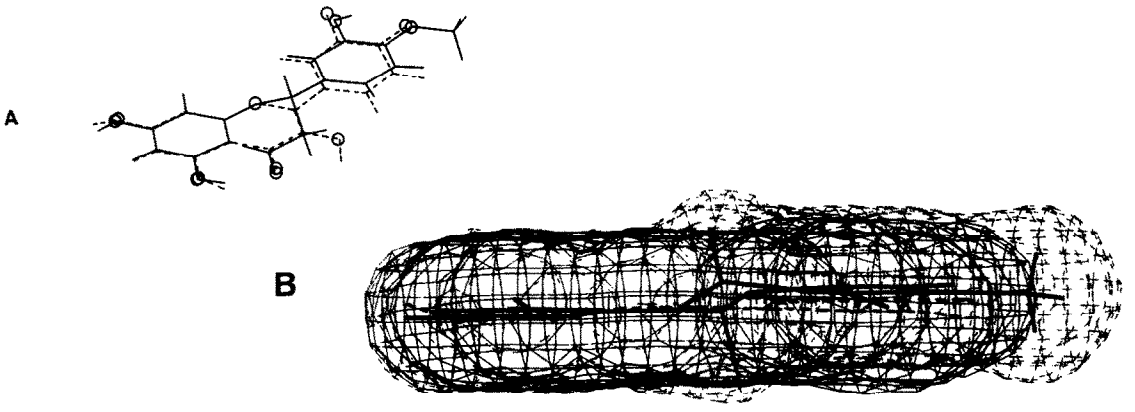

Fig. 7. (A) Molecular conformation of hesperetin (solid line) and quercetin (dashed line), illustrating the similarity in overall shape of these two flavonoids. (B) Superposition of the planar quercetin, viewed edge on (dashed line), on that of the flavanone hesperetin (solid line) showing the van der Waals surface of hesperetin. Note that the presence of the 4′-methoxy extends the length of the structure beyond that of quercetin and that the sofa conformation of the pyran ring of hesperetin causes a slight thickening of the structural profile compared to the planar quercetin.

Kinetic analysis of flavonoid inhibition with respect to components of the catalytic subunit showed that fisetin, the most potent flavonoid, was a competitive inhibitor with respect to ATP binding, and noncompetitive with respect to protein substrate. The same pattern of inhibition was seen with luteolin. The extent of inhibition of PKC by fisetin was not influenced by preincubating different combinations of flavonoid, ATP and enzyme, suggesting that inhibition was not dependent upon formation of a slow-forming ATP–enzyme or substrate–enzyme complex.

To understand the mechanism of action of these flavonoids as inhibitors of PKC activation, the structural properties of the fifteen flavonoids tested in this system were compared. This biochemical analysis revealed that the most potent flavonoids were characterized by a 7-hydroxyl benzopyrone ring system with either a 3- or 5-hydroxy substituent and a 3′,4′-dihydroxy-2-phenyl ring, as with quercetin (Fig. 1). Those compounds with partial potency fell into two general structural types: chalcones with an open ring structure (phloretin and chalcone) and flavones with incomplete or methoxylated ring substitution patterns (apigenin and isorhamnetin). The least potent flavonoids, e.g. taxifolin and naringin, were characterized by either an unsaturated flavanone ring system and inappropriate ring substitution or glycosylation of key positions.

Analysis of the stereochemical characteristics of the flavonoids based on crystallographic data showed that their conformations fall into two classes dependent upon the state of saturation of the C2–C3 bond. The observed coplanar structure of quercetin [21] (Fig. 7A) is representative of the class with an unsaturated C2–C3 bond. Structural comparison shows that the conformational preference of flavanones is to have a sofa pyran ring with an equatorial phenyl ring twisted about 45° with respect to the benzopyran ring system [2], whereas flavones such as quercetin are coplanar. The structure of hesperetin reported herein is unusual: the phenyl ring is coplanar with the benzopyran ring system and the combined displacement of the C2 atom in a sofa conformation and the 27° twist of the phenyl ring causes the two rings to be coplanar as observed in the active flavones (Fig. 7). Correlation of the biochemical and structural data suggest that: (1) methylation of the 4′ position of hesperetin contributes to its inactivity as a PKC inhibitor because, as shown in Fig. 7B, this group extends the length of the molecule and also disrupts the di-keto tautomerization and hydrogen bonding of the 3′, 4′-dihydroxy groups, (2) the results further imply, based on these limited data, that a 2-phenyl flavone is required for potency as the 3-phenyl isoflavonoid is inactive, and (3) the partial activities of the flavones, apigenin and isorhamnetin, which are also expected to have a coplanar conformation

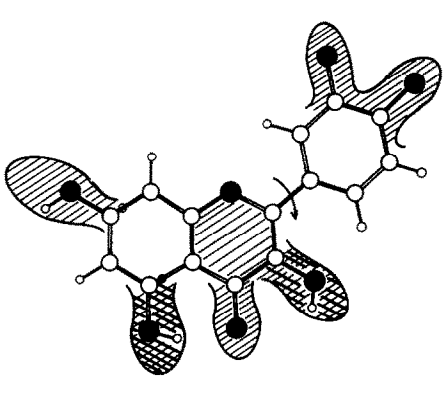

Fig. 8. Model of the principal flavonoid structural features required for maximum PKC inhibition. The diagonally shaded areas are essential features, i.e. a flavone pyrone ring, a coplanar phenyl ring (Torsion Angle C3–C2–C1′–C2′ = 0°) and 3′,4′,7-hydroxyls. The cross-hatched areas are those hydroxyl substituents that are nonessential to PKC inhibition.

similar to quercetin, result from either incomplete or inappropriate substitutions at key recognition sites; thus, the absence of the 3′-hydroxy in apigenin and the blocking of the 3′-position by a methoxy group in isorhamnetin account for their partial potencies.

Based on this analysis we propose a model of the principal structural features required for PKC inactivation by flavonoids (Fig. 8). The model includes a planar benzopyrone ring system with a 7-hydroxy and a coplanar 2-(3′,4′-dihydroxy)-phenyl ring.

The flavonoids may prove useful as (1) biochemical tools with which to examine the physiological role of phosphorylation induced by PKC, (2) affinity ligands in the purification of PKC, and (3) pharmacological modulators of PKC activity *in vivo*.

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REFERENCES

1. Harborne JB, Nature, distribution, and function of plant flavonoids. In: *Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological and Structure–Activity Relationships* (Eds. Cody V, Middleton E and Harborne J), pp. 15–24. Alan R. Liss, New York, 1986.
2. Laychock SG, The biochemistry of cell activation as related to the putative actions of flavonoids. In: *Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological and Structure–Activity Relationships* (Eds. Cody V, Middleton E and Harborne J), pp. 215–230. Alan R. Liss, New York, 1986.
3. Kato R, Nakadate T, Yamamoto A and Sugimura T, Inhibition of 12-*O*-tetradecanoylphorbol-13-acetate-induced tumor promotion and ornithine decarboxylase activity by quercetin: Possible involvement of lipoxygenase inhibition. *Carcinogenesis* **4**: 1301–1305, 1983.
4. Nishino H, Naito A, Iwashima A, Tanaka K-I, Matsuura T, Fujiki H and Sugimura T, Interaction between quercetin and Ca^{2+}-calmodulin complex: Possible mechanism for anti-tumor promoting action of the flavonoid. *Jpn J Cancer Res (Gann)* **75**: 311–316, 1984.
5. Middleton E Jr and Drzewiecki G, Flavonoid inhibition of human basophil histamine release stimulated by various agents. *Biochem Pharmacol* **33**: 3333–3338, 1984.
6. Middleton E Jr, Fujiki H, Savliwala M and Drzewiecki G, Tumor promoter-induced basophil histamine release. *Biochem Pharmacol* **36**: 2048–2052, 1987.
7. Castagna M, Takai Y, Kaibachi K, Sano K, Kikkawa U and Nishizuk Y, Direct activation of calcium activated phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J Biol Chem* **257**: 7847–7851, 1982.
8. Fujiki H, Tanaka Y, Miyake R, Kikkawa U, Nishizuka Y and Sugimura T, Activation of calcium-activated phospholipid-dependent protein kinase (protein kinase C) by new classes of tumor promoters: teleocidin and debromoaplysiatoxin. *Biochem Biophys Res Commun* **120**: 339–343, 1984.
9. Gschwendt M, Horn F, Kittstein W and Marks F, Inhibition of the calcium- and phospholipid-dependent protein kinase activity from mouse brain cytosol by quercetin. *Biochem Biophys Res Commun* **117**: 444–447, 1983.
10. Horiuchi T, Fujiki H, Hakii H, Suganuma M, Yamashita K and Sugimura T, Modulation of phorbol ester receptors in mouse skin by application of quercetin. *Jpn J Cancer Res (Gann)* **77**: 526–531, 1986.
11. Kikkawa U, Minakuchi R, Takai Y and Nishizuka Y, Calcium-activated, phospholipid-dependent protein kinase (protein kinase C) from rat brain. *Methods Enzymol* **99**: 288–298, 1983.
12. Segel IH, *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, pp. 71 and 217. John Wiley, New York, 1975.
13. Bradford MM, A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
14. Wise BC, Glass DB, Chou C-HJ, Raynor R-L, Katoh N, Schatzman RC, Turner RS, Kibler RF and Kuo JF, Phospholipid-sensitive Ca^{2+}-dependent protein kinase from heart. II. Substrate specificity and inhibition by various agents. *J Biol Chem* **257**: 8489–8495, 1982.
15. Kikkawa U, Takai Y, Minakuhci R, Inohara S and Nishizuka Y, Calcium-activated, phospholipid-dependent protein kinase from rat brain. Subcellular distribution, purification and properties. *J Biol Chem* **257**: 13341–13348, 1982.
16. Ashendel CL, Staller JM and Boutwell RK, Protein kinase activity associated with a phorbol ester receptor purified from mouse brain. *Cancer Res* **43**: 4333–4337, 1983.
17. German G, Main P and Woolfson MM, The application of phase relationships to complex structures. III. The optimum use of phase relationships. *Acta Crystallogr* **A27**: 368–376, 1971.
18. De Titta GT, Edmonds JW, Langs DA and Hauptman H, Use of negative quartet cosine invariants as a phasing figure of merit: NQEST. *Acta Crystallogr* **A31**: 472–479, 1975.
19. Ibers JA and Hamilton WC (Eds.), *International Tables for X-ray Crystallography*. Kynoch Press, Birmingham, 1974.
20. Rossi M, Rickles LF and Halpin WA, The crystal molecular structure of quercetin: A biologically active and naturally occurring flavonoid. *Bioorgan Chem* **14**: 55–69, 1986.
21. Auf'mkolk M, Koehrle J, Hesch RD, Ingbar SH and Cody V, Inhibition of rat liver iodothyronine deiodinase. Interaction of aurones with the iodothyonine ligand-binding site. *Biochem Pharmacol* **35**: 2221–2227, 1986.
22. Glusker JP and Rossi M, Molecular aspects of chemical carcinogens and bioflavonoids. In: *Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological and Structure–Activity Relationships* (Eds. Cody V, Middleton E and Harborne J), pp. 395–410. Alan R. Liss, New York, 1986.
23. Nishizuka Y, Studies and perspectives of protein kinase C. *Science* **233**: 305–312, 1986.