Biochemical Pharmacology, Vol. 33, No. 3, pp. 357–363, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00
© 1984 Pergamon Press Ltd.

INHIBITION *IN VITRO* OF PLATELET AGGREGATION AND ARACHIDONIC ACID METABOLISM BY FLAVONE*

RICHARD L. MOWER, RAFFELE LANDOLFI and MANFRED STEINER†
Division of Hematology, Brown University's Memorial Hospital, Pawtucket, RI 02860 U.S.A.

(*Received* 4 *April* 1983; *accepted* 30 *June* 1983)

Abstract—The effects of flavone on platelet aggregation and arachidonic acid (AA) metabolism were tested *in vitro*. When incubated at a concentration of 50 μM, flavone completely suppressed platelet aggregation induced by 150 μM AA in thirty-six out of forty-three subjects tested. A lower concentration (10 μM) was effective in about 50% of the donors. Flavone also inhibited the second wave of aggregation induced by epinephrine and ADP. Platelet thromboxane formation, estimated both by radioimmunoassay measurements and by studies of ^{14}C-labeled AA metabolism, was depressed by flavone. Flavone-treated platelets preferentially utilized [^{14}C]AA for the lipoxygenase pathway while cyclo-oxygenase activity was depressed. Adenosine 3':5'-cyclic monophosphate (cAMP) was measured in flavone-treated and control platelets. While their baseline levels were similar, flavone-treated platelets showed a lower stimulation of cAMP induced by prostacyclin (PGI_2) than did controls. Phosphodiesterase activity was not affected by flavone as judged from the decay rates of PGI_2-stimulated cAMP levels. From these findings we conclude that the antiaggregating activity of flavone is not a consequence of changes in platelet cAMP but is due to inhibition of cyclo-oxygenase.

Flavonoids are a large family of plant pigments whose chemical structures are based on that of flavone. Several of these compounds have been reported to inhibit platelet aggregation [1, 2] and adhesion to glass [3]. The antiplatelet activity of flavonoids, potentially useful for clinical purposes, could result from an elevation of platelet cAMP, a potent inhibitor of platelet aggregation. These compounds, in fact, have been reported to be capable of raising cellular levels of cAMP through an inhibition of cAMP-phosphodiesterase [4–7]. Such an effect has been shown for the flavonoid quercetin. This compound elevated platelet cAMP and thus was found to potentiate the antiaggregating effect of prostacyclin (PGI_2) [8]. In addition, quercetin inhibited the Ca^{2+} influx in inside-out red cell membrane vesicles [9, 10] and also in certain other cells [11–13], presumably by blocking Ca^{2+}-dependent ATPase. In rat kidney, flavonoids are known to inhibit prostaglandin synthesis [14]. These observations prompted us to test the effects of flavone, the principal protagonist of this group of compounds, on platelet arachidonic acid metabolism and aggregation, as well as on platelet cAMP levels.

METHODS

Preparation of platelets and aggregation studies. Platelets were obtained from volunteers who were selected on the basis of abstention from any medication for 14 days prior to phlebotomy. Platelet rich plasma (PRP) was separated from citrate phosphate dextrose (CPD) anticoagulated blood at 260 *g* for 15 min. Aggregation of 0.45 ml PRP was monitored turbidimetrically on a Chrono-Log aggregometer connected to a recorder. All aggregations were carried out at 37° with PRPs having platelet counts between 2.5 and 3×10^8/ml. Platelet aggregability was screened with arachidonic acid (AA) and epinephrine. The PRPs of all subjects included in this study had threshold aggregating concentrations of AA ranging between 100 and 120 μM and complete aggregation responses to 6 μM epinephrine. A 1 mM stock solution of AA was prepared by sonicating 3.1 mg of arachidonic acid into 10 ml of degassed 0.14 M NaCl, 10 mM Tris–HCl, pH 7.4 (TBS), containing 0.25% bovine serum albumin (BSA) and 20 μM butylated hydroxytoluene (BHT). Bovine tendon collagen was solubilized with acetic acid, and dilutions from a stock solution of 5 mg/ml were utilized for measurement of collagen-induced aggregation.

Flavone was prepared as a 1 mM solution by sonicating the drug in TBS containing 0.5% BSA. To measure the effect of flavone on platelet aggregation, PRPs were incubated for 10 min at room temperature with various concentrations of flavone. The aggregation tracings were compared to those of controls that were incubated with equivalent amounts of TBS containing 0.5% BSA.

Measurement of thromboxane production. Thromboxane A_2 (TxA_2) production by human platelets was tested by radioimmunoassay (RIA) of TxB_2 (stable metabolite of TxA_2) in the supernatant fraction of thrombin-aggregated, washed platelets before and after incubation with flavone. Platelets obtained from fifteen volunteers were separated by gel filtration on Sepharose CL-2B columns equilibrated with TBS. After adjusting platelet counts to 1.8×10^8 cells/ml, stirred platelet suspensions were aggregated with thrombin (0.1 units/ml). After 5 min, the

* Supported by research grant HL 13587 of the National Heart, Lung and Blood Institute.

† Address all inquiries about this paper to: Manfred Steiner, M.D., Ph.D., Division of Hematologic Research, The Memorial Hospital, Pawtucket, RI 02860.

platelets were centrifuged at 4000 *g* for 2 min. Aliquots of the supernatant fraction were rapidly frozen and kept at −70° until used. The radioimmunoassay of TxB_2 was performed with an RIA kit obtained from the New England Nuclear Corp. Boston, MA. The assay was sensitive over the range of 10 to 250 pg/0.1 ml.

Evaluation of oxidative metabolism of arachidomic acid in platelets. Arachidonic acid metabolism was studied by both prelabeling and pulse-labeling techniques. For the latter, platelets were washed once in TBS/77 mM EDTA (98:2, v/v) and then purified by a modification of the procedure of Hamberg *et al.* [15] in which EDTA and residual plasma were removed by gel filtration on Sepharose CL-2B. Flavone was prepared as for aggregation studies. Usually the compound was preincubated for 5 min at room temperature with platelet suspensions. Aggregation was started by addition of 0.5 ml of 30 mM $CaCl_2$ containing 5 N.I.H. units of bovine thrombin and 0.1 μCi of [1-^{14}C]arachidonic acid (sp. act. 55.8 mCi/mmol; New England Nuclear) to 4.5 ml of platelet suspension. After 3 min at 37°, the reaction was stopped by addition of an equal volume of 1 N HCl and rapid extraction/partition with 10 vol. of chloroform containing 10 μM BHT. The extracts were flash-evaporated under nitrogen *in vacuo* and applied to thin-layer chromatography (TLC) plates coated with hard layer silica gel containing inorganic binder with UV 254 phosphor (Supelco, PA). After a 45-min development in isooctane–ethyl acetate–acetic acid–H_2O (50:50:2:2, upper layer), the plates were scanned for radioactivity on a Packard 7220 scanner. Areas of interest on TLC plates were scraped into Aquasol II (New England Nuclear) and counted in a liquid scintillation spectrometer. Arachidonic acid and the AA metabolites, 12-L-hydroxy-5,8,10-heptadectrienoic acid (HHT), 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE) and TxB_2, as well as phospholipids (PL) and cholesterol ester (CE), were identified on the basis of their R_f values using appropriate standards.

A modified prelabeling technique was used to incorporate [1-^{14}C]AA into platelets prior to aggregation [16, 17]. During a 10-min interval, 1.0 to 1.5 μCi of [1-^{14}C]AA (sp. act. 55.8 mCi/mmol) in 1 ml TBS/EDTA containing 0.5% BSA was added to 0.05-ml aliquots of concentrated platelet suspensions (1.5×10^9 ml) at 25°. To achieve maximal (78%) incorporation of the labeled fatty acid, a further incubation of 30 min was found necessary [18]. The total labeling period was 40 min, at the end of which BSA, residual plasma, and unincorporated AA were removed by gel filtration. Aggregation was induced as in pulse-labelling studies except that no further [1-^{14}C]AA was added.

Cyclic AMP assay. Cyclic AMP was measured in control and 50 μM flavone-treated platelets. Suspensions were prepared of platelets separated by gel filtration of PRP over Sepharose CL-2B columns and equilibrated with Lindon's buffer [19] containing 0.5% BSA. Aliquots of platelet suspensions, 1 ml in volume, obtained before and at 1, 15 and 25 min after addition of PGI_2 at a final concentration of 1 nM, were precipitated by adding an equal volume of 10% trichloroacetic acid containing [^{3}H]cAMP as internal standard. The precipitates were extracted three times with 5 vol. of diethyl ether. The aqueous phases of the extracts were lyophilized and kept at −70° until the assays could be performed. Cyclic AMP was measured by radioimmunoassay using a [^{125}I]cAMP RIA kit (New England Nuclear) and following the acetylation technique described by Steiner *et al.* [20]. All results were corrected for recovery which was estimated by the internal standard method.

Statistical methods. The significance of the change in platelet AA metabolism before and after treatment with flavone was evaluated by paired *t*-test analysis [21].

Materials. Solvents and reagents were purchased from the Fisher Scientific Co., Pittsburgh, PA. Specialty chemicals were obtained through the Sigma Chemical Co., St. Louis, MO, except arachidonic acid (Nu-Chek Prep, Elysian MN) and bovine thrombin (Parke-Davis, Detroit, MI). Fenwal CPD blood bags were used for larger donations. Sepharose CL-2B was a product of Pharmacia, Piscataway, NJ. Gifts of AA metabolites were gratefully accepted from Dr. John Pike (Upjohn, Kalamazoo, MI).

RESULTS

Platelet aggregation. The effect of flavone on the ability of 150 μM arachidonic acid to induce platelet aggregation was tested using blood from forty-three donors. When PRPs were incubated with 50 μM flavone for 10 min at room temperature, platelet aggregation was completely suppressed in the PRPs of thirty six subjects. Higher concentrations of flavone were effective in the PRPs from another five individuals, whereas platelet aggregation in the PRPs of the remaining two donors was not inhibited by concentration of flavone as high as 200 μM. The percentage of PRPs responding at various concentrations of flavone is reported in Table 1. In the PRPs of some individuals the inhibition by the standard concentration of 50 μm flavone could be overcome by increasing the AA concentration to 200 μM. This was observed only with the PRPs of four subjects in which the minimum flavone concentration that inhibited was >10 μM. The effects of flavone on ADP-, epinephrine-, and collagen-induced platelet aggregation were studied in PRPs from six donors. At a concentration of 50 μM, flavone inhibited the second wave of aggregation due to 2 μM ADP and 6 μM epinephrine (Fig. 1). Platelet aggregation induced by collagen was less affected by flavone. A moderate (approximately 20%) reduction of maximal aggregation was detectable, but only when low doses (<3 μg/ml) of collagen were used.

Products of oxidative conversion of arachidonic acid. All pulse-labeled platelets that were pretreated with flavone showed a depression of the products (TxB_2 and HHT) of the cyclo-oxygenase pathway, while HETE, a metabolic product of lipoxygenase, was increased (Fig. 2). As direct inhibition of cyclo-oxygenase and direct stimulation of lipoxygenase could have produced this pattern, we investigated the effect of flavone on platelets in which the cyclo-oxygenase pathway was first inhibited by 10 μM indomethacin. In this situation flavone did not

Table 1. Inhibition by flavone of platelet aggregation induced by 150 μM arachidonic acid: dose–response relation

Concentration of flavone (μM)	5	10	25	35	50	100
% PRPs responding*	25.5	48.8	72	76	83	95

* Blood was donated by 43 individuals, each of whose PRP was used for dose–response measurements.

increase HETE production, and AA utilization was reduced in most of the individuals studied (data not shown).

The results obtained with platelets of seventeen subjects are summarized in Table 2. The incorporation of [1-^{14}C]AA into individual oxidative metabolites in PRP showed considerable variability from donor to donor. For this reason we used each subject as his own control in assessing the effect of flavone. Paired *t*-tests were highly significant for all but the change in AA ($P < 0.0005$). Total incorporation of [^{14}C]AA into platelets did not differ significantly between controls and flavone-treated cells. AA utilization tended to decrease but the sample size was too small to make the flavone-induced change significant.

To rule out the possibility that flavone interfered with the uptake of AA by platelets, we studied the effect of flavone on platelets prelabeled with AA (Fig. 3). The results obtained in PRPs of four donors are reported in Table 3. As observed in pulse-label experiments, flavone depressed production of TxB_2 and HHT and tended to increase that of HETE. However, the reduction of AA metabolism resulted in an increase of radioactivity in the PL fraction rather than in AA; in one individual in whom flavone failed to inhibit AA-induced aggregation, no significant effect on AA metabolism was detected either.

The effect of flavone on TxB_2 production by thrombin-stimulated (0.1 units/ml) platelets was also studied by RIA. Out of fifteen donors PRPs tested, only one failed to show a decrease of Tx formation. When platelets were treated with 50 μM flavone, various degrees of inhibition (mean 62%) were found in the other subjects. As shown in Fig. 4, flavone was, in general, equally inhibitory in PRPs with high and low basal Tx formation.

Cyclic AMP measurements. Radioimmunoassay of cAMP showed similar values in control and flavone-treated platelets (Fig. 5). Stimulation of cAMP by PGI_2 produced the expected increase of cyclic nucleotides which reached eight times the baseline value in control platelets. In flavone-treated platelets, PGI_2 was not as effective in raising cAMP (about 4-fold). The decrease of cAMP following

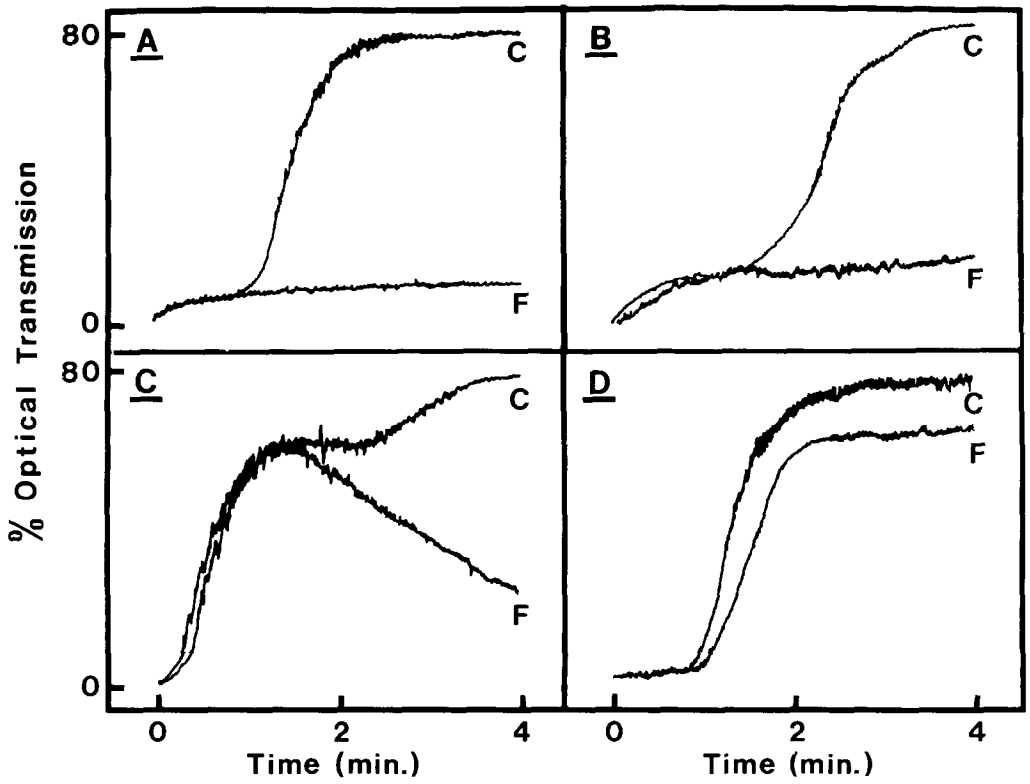

Fig. 1. Typical aggregation patterns of control (C) and 50 μM flavone (F)-treated platelets. Aggregation was induced by 150 μM AA (A), 6 μM epinephrine (B), 2 μM ADP (C) and 2 μg/ml collagen (D).

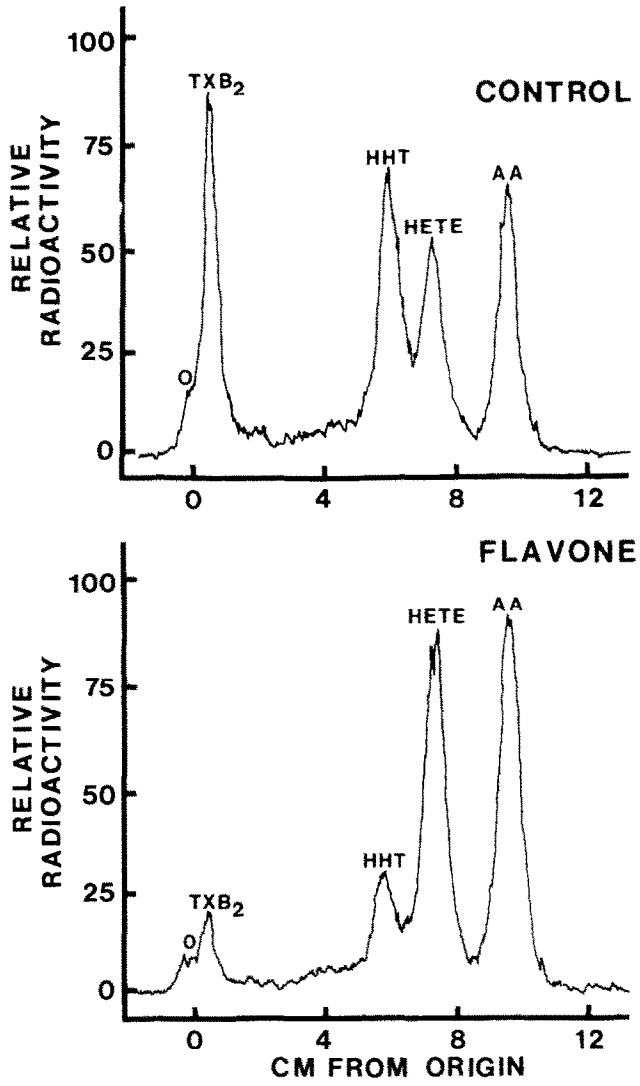

Fig. 2. Radiochromatograph scans of oxidative metabolites of AA extracted from control (upper panel) and 50 μM flavone-treated [1-^{14}C]arachidonic acid (lower panel). Conditions of incubation and extraction procedures are described under Methods. Abbreviations: AA, arachidonic acid; HETE, 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid; HHT, 12-L-hydroxy-5,8,10-heptadectrienoic acid: and TxB_2, thromboxane B_2.

Table 2. Distribution of radioactivity derived from [^{14}C]arachidonic acid in control and 50 μM flavone-treated human platelets*

	No. of expt.	Percent of total radioactivity (mean ± S.E.M.)			
		AA	HETE	HHT	TxB_2
Control	17	25.3 ± 4.5	26.1 ± 2.2	28.2 ± 2.3	18.4 ± 2.2
Flavone, 50 μM	17	35.6 ± 4.0	40.4 ± 3.1†	17.0 ± 1.1†	5.4 ± 1.1†

* Each experiment was run with its own control. Abbreviations: AA, arachidonic acid; HETE, 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid; HHT, 12-L-hydroxy-5,8,10-heptadectrienoic acid; and TxB_2, thromboxane B_2.

† Statistical significance ($P < 0.0005$) as derived by paired t-test analysis.

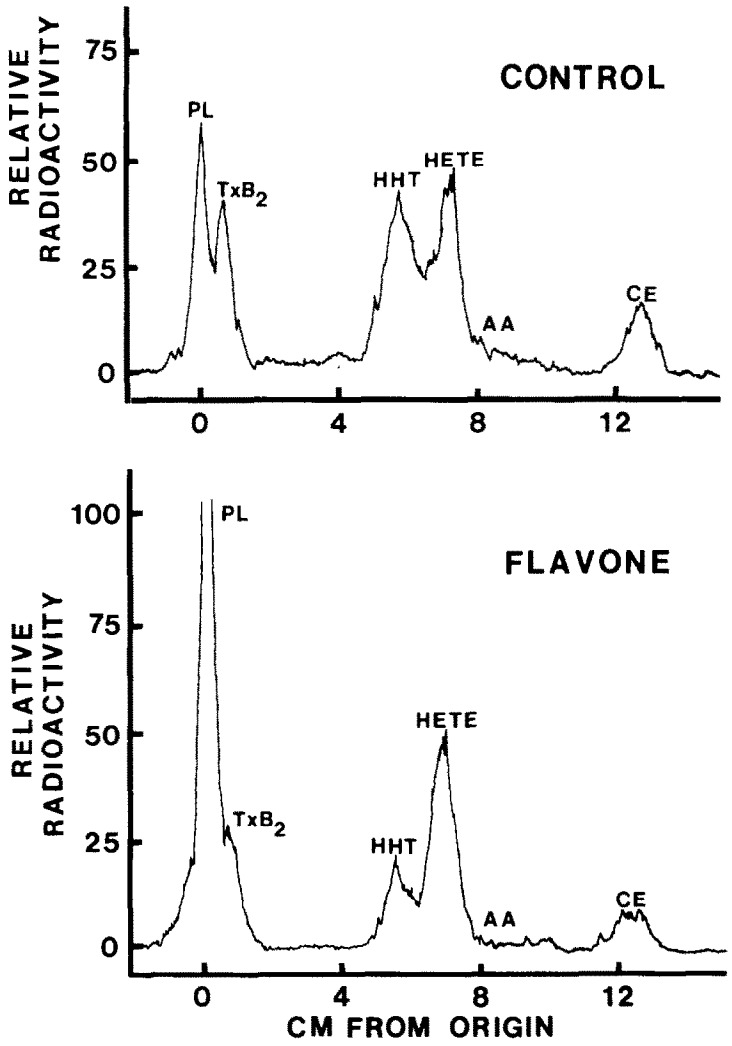

Fig. 3. Radiochromatograph scans of oxidative metabolites of AA extracted from control (upper panel) and 50 μM flavone-treated (lower panel) platelets. Platelets were prelabeled with [1-^{14}C]arachidonic acid. Conditions of incubation and extraction procedures are described under Methods. Abbreviations: PL, phospholipids; CE, cholesterol ester; AA, arachidonic acid; HETE, 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid; HHT, 12-L-hydroxy-5,8,10-heptadetrienoic acid; and TxB_2, thromboxane B_2.

Table 3. Distribution of radioactivity derived from [^{14}C]arachidonic acid in control and flavone-treated human platelets*

Expt.	No.	Percent of total radioactivity					
		CE	AA	HETE	HHT	TxB_2	PL
1.	Control	9.2	3.7	19.4	18.3	16.2	33.2
	Flavone, 50 μM	9.7	1.8	20.9	10.6	6	51
2.	Control	13.9	2.8	22.8	13.3	8.2	39
	Flavone, 50 μM	12.3	2.8	26.6	5.4	2.4	50.5
3.	Control	5.9	3.9	32	27	18.2	13
	Flavone, 50 μM	4	1.2	36.6	13	9.2	36
4.	Control	7.7	15	17.5	12.6	10.2	37
	Flavone, 50 μM	5.4	16.6	15.4	13.3	9.8	39.5

* Prelabel experiments (see Methods). Abbreviations: CE, cholesterol ester; PL, phospholipids; AA, arachidonic acid; HETE, 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid; HHT, 12-L-hydroxy-5,8,10-heptadextrienoic acid; and TxB_2, thromboxane B_2.

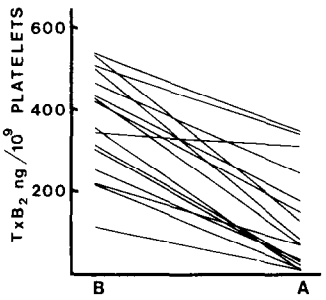

Fig. 4. Effect of 50 μM flavone on TxB_2 generation by thrombin aggregated human platelets. Production of TxB_2 in fifteen subjects was measured by RIA before (B) and after (A) incubation with flavone (see Methods).

stimulation was measured over a 25-min interval. The decay curves were similar in both control and flavone-treated platelets.

DISCUSSION

Flavone appeared to have a rather potent inhibitory effect on AA-induced platelet aggregation, being active in the PRPs of about 50% of the donors who were studied, at concentrations as low as 10 μM. Flavone inhibited the second wave of aggregation induced by ADP and epinephrine, but had less effect on collagen-induced aggregation. The latter is not surprising because the platelet response to collagen shows low sensitivity to most antiaggregating agents available including aspirin [22]. This is probably due to the ability of collagen to induce platelet aggregation via several different mechanisms [23]. The same holds true for ADP, but in this case the prostaglandin pathway seems to play the major role.

Two possible mechanisms of inhibition of platelet function by flavone were investigated. Elevation of cAMP, as described for certain flavonoids in other cells [11–13], did not play a significant role in the suppression of platelet aggregability. This is based on the observation that flavone did not affect basal platelet cAMP levels. Moreover, flavone clearly decreased the PGI_2-induced rise in cAMP. It is especially noteworthy that quercetin, a structurally related flavonoid, has been reported recently to exhibit effects opposite to those we found with flavone [8]. While the potentiation of the PGI_2-induced increase of platelet cAMP by quercetin was probably the result of an inhibition of phosphodiesterase, the effect of flavone on cAMP was most likely a consequence of interference with the production, rather than the catabolism, of the cyclic nucleotide. Further studies will be required to confirm or refute this hypothesis.

The effects of flavone on AA metabolites, as judged by the results of prelabel and pulse-label studies, were qualitatively similar to those described for vitamin E [18] although flavone was far more potent; the structural similarities between flavone and vitamin E, both compounds having a chromane ring, probably account for this effect.

The variability in the response of different PRPs to the drug was also an interesting finding of our investigation. At this time, we have no evidence of a difference between sensitive and resistant platelets. The platelets of one donor were completely resistant to the antiaggregating activity of flavone, and the labeling study also showed no effect of the drug on cyclo-oxygenase activity. Furthermore, in this one instance the basal activity of the enzyme was quite low. This finding, although isolated and thus of limited value, is contrary to the seemingly plausible hypothesis that flavone is less effective in platelets with a high basal thromboxane production.

The abundance of naturally occurring flavonoids and their increasing use as pharmacologic agents make it desirable and necessary to investigate their "side effects". The effect of flavone on cyclo-oxygenase activity shows that elevation of platelet cAMP, described for quercetin [8], is not the only mechanism of platelet inhibition by flavonoids. The coexistence of at least two different modes of inhibition is of practical as well as scientific interest.

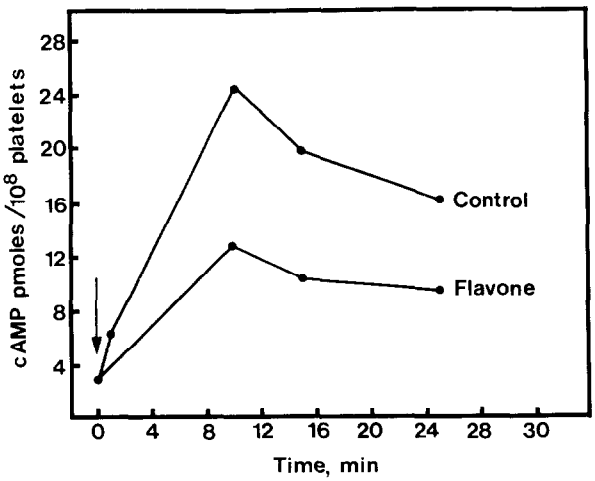

Fig. 5. Effect of 50 μM flavone on cAMP levels of platelets stimulated with 1 nM PGI_2. Cyclic AMP was measured by RIA as described under Methods. The arrow indicates addition of prostacyclin. Each point represents the mean of two measurements.

REFERENCES

1. J. Pollock and H. Heath, *Biochem. Pharmac.* **24**, 397 (1975).
2. J. W. Ten Cate, N. J. Van Haeringen, J. Gerritsen and E. Glasius, *Clin. Chem.* **19**, 31 (1973).
3. E. Sempinska, B. Kostka, M. Krolikowska and E. Kalisiak, *Pol. J. Pharmac. Pharmacy* **29**, 7 (1977).
4. A. Beretz, R. Anton and J. C. Stoclet, *Experientia* **34**, 1054 (1978).
5. J. E. Ferrel, P. G. D. Chang Ling, G. Loew, R. King, J. M. Mansour and T. E. Mansour, *Molec. Pharmac.* **16**, 556 (1979).
6. M. Ruckstuhl, A. Beretz, R. Anton and Y. Landry, *Biochem. Pharmac.* **28**, 535 (1979).
7. M. Ruckstuhl and Y. Landry, *Biochem. Pharmac.* **30**, 697 (1981).
8. A. Beretz, A. Stierle, R. Anton and J. P. Cazenave, *Biochem. Pharmac.* **31**, 3597 (1982).
9. B. Sarkadi, I. Szasz and G. Gárdos, *Biochim. biophys. Acta* **598**, 326 (1980).

10. A. Wuthrich and H. J. Schatzman, *Cell Calcium* **1**, 21 (1980).
11. C. M. S. Fewtrell and B. D. Gomperts, *Biochim. biophys. Acta* **469**, 52 (1977).
12. J. P. Bennet, B. D. Gomperts and E. Wollenweber, *Arzneimittel-Forsch.* **31**, 433 (1981).
13. V. Shoshan, K. P. Campbell, D. H. MacLennan, W. Frodis and B. A. Britt, *Proc. natn. Acad. Sci. U.S.A.* **77**, 4435 (1980).
14. J. Baumann, F. V. Bruchausen and G. Wurm, *Naunyn-Schmiedeberg's Archs Pharmac.* **307**, 73 (1979).
15. G. Hamberg, J. Svensson, T. Wakabayashi and B. Samuelsson, *Proc. natn. Acad. Sci. U.S.A.* **71**, 345 (1974).
16. R. Mower and M. Steiner, *Prostaglandins* **24**, 137 (1982).
17. S. J. Jubeliere, R. Russel, V. Vaillancourt and D. Deykin, *Blood* **57**, 728 (1980).
18. R. Mower and M. Steiner, *Prost. Leuk. Med.* **10**, 389 (1983).
19. K. M. Fine, P. C. Ashbrook, L. P. Brigden, J. E. Maldonado and P. Didisheim, *Am. J. Path.* **84**, 11 (1976).
20. A. L. Steiner, C. W. Parker and D. M. Kipnis, *J. biol. Chem.* **247**, 1106 (1972).
21. A. Goldstein, *Biostatistics*, p. 59. Macmillan, New York (1964).
22. M. A. Packham, M. A. Guccione, P. L. Chang and J. F. Mustard, *Am. J. Physiol.* **225**, 38 (1973).
23. B. B. Vargaftig, M. Chignard, J. P. LeCouedic and J. Benveniste, *Acta med. scand.* (Suppl.) **643**, 23 (1980).