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Extracts of feverfew may inhibit platelet behaviour via neutralization of sulphydryl groups

S. HEPTINSTALL*, W. A. GROENEWEGEN, P. SPANGENBERG† AND W. LOESCHE†

Department of Medicine, University Hospital, Queen's Medical Centre, Nottingham NG7 2UH, UK, and †Institute of Pathological Biochemistry, Medical Academy of Erfurt, Erfurt, GDR

It has been suggested that extracts of feverfew may inhibit platelet behaviour via effects on platelet sulphydryl groups. In the present study we have obtained evidence for such a mode of action. (i) Compounds that contain sulphydryl groups such as cysteine and *N*-(2-mercaptopropionyl)glycine prevented the inhibition of platelet behaviour by feverfew. (ii) Feverfew and parthenolide (one of the active components of feverfew) dramatically reduced the number of acid-soluble sulphydryl groups in platelets. This effect occurred at concentrations similar to those that inhibited platelet secretory activity. (iii) Feverfew itself did not induce the formation of disulphide-linked protein polymers in platelets but polymer formation occurred when aggregating agents were added to feverfew-treated platelets. (iv) Feverfew evoked changes in the metabolism of arachidonic acid that were similar to those observed in glutathione-depleted platelets.

Extracts of feverfew (*Tanacetum parthenium*) inhibit both platelet aggregation and release of 5-hydroxytryptamine (5-HT) from platelets (Heptinstall et al 1985) and such inhibition may be relevant to the beneficial effects of the herb in migraine and other conditions (Johnson et al 1985; Editorial 1985). Fractionation of an extract of feverfew followed by analysis of the fractions obtained, indicated that the components of feverfew that are responsible for its anti-secretory activity are sesquiterpene lactones (Groenewegen et al 1986). Examples of sesquiterpene lactones that are found in feverfew are parthenolide, 3-β-hydroxyparthenolide, seco-tanapartholide A, canin and artecanin, all of which contain an α-methylenebutyrolactone unit as an integral part of their chemical structure. The activated methylene group in this unit renders it susceptible to nucleophilic attack via Michael addition, and it has been suggested that the anti-secretory activity displayed by compounds that contain this unit may be consequent to interaction with molecules that contain sulphydryl groups (Groenewegen et al 1986).

The purpose of this investigation was to gain evidence for such a mechanism of action. Four different approaches were used. First, compounds that contain sulphydryl groups were examined for their capacity to neutralize the inhibitory effects of feverfew extract on platelet aggregation and 5-hydroxytryptamine release. Second, the effects of feverfew extract and of parthenolide on acid soluble (non-protein, mainly reduced glutathione) and acid-insoluble (protein) sulphydryl groups present in platelets were determined, and the amounts of feverfew extract or parthenolide that were needed to reduce the number of sulphydryl groups were compared with the amounts that were required for anti-secretory activity. Third, platelets were treated with feverfew extract in the absence and presence of platelet aggregating agents and the effects of the treatments on the protein composition of platelets were determined by gel electrophoresis. Finally, the effects of feverfew extract on the ability of platelets to metabolize exogenous [^{14}C]arachidonic acid ([^{14}C]AA) were investigated.

* Correspondence.

MATERIALS AND METHODS

Preparation of feverfew extracts

Leaves that had been taken from actively growing plants in the Department of Botany, University of Nottingham, were air-dried and extracted using phosphate-buffered saline (250 mg mL^{-1} of PBS). The solid material was removed by filtration and the filtrate was then extracted with chloroform. The organic phase was evaporated to dryness and the solid residue was suspended in PBS. The volume of PBS used at this stage was the same as the volume of filtrate obtained after the first extraction. Finally, any material that remained undissolved was again removed by filtration.

Preparation of a solution of parthenolide

A sample of parthenolide (a gift from Dr P. J. Hylands) was dissolved in 2% (v/v) ethanol in PBS at

a concentration of 1 mM. This was further diluted using 2% ethanol in PBS as required. When parthenolide was added to platelet preparations (see below) the final concentration of ethanol was always 0·33% (v/v).

Blood collection and platelet preparation

Aliquots (9 mL) of venous blood from healthy volunteers were collected into tubes that contained 3·8% (w/v) trisodium citrate dihydrate (1 mL) as anticoagulant and platelet-rich plasma (PRP) was prepared by centrifugation (Heptinstall & Fox 1983). When measurements of the platelet release reaction were to be performed, [^{14}C]5-hydroxytryptamine creatinine sulphate ([^{14}C]5-HT, 6 μL of a solution of specific activity 55 mCi mmol^{-1}, 50 μCi mL^{-1} from Amersham International) was added to the anticoagulant before the blood was collected and centrifuged. In the PRP that was obtained, more than 90% of the [^{14}C]5-HT was found to be intracellular (Heptinstall & Fox 1983).

In some experiments platelets were washed and suspended in a physiological salt solution before investigation. For this, blood was collected into isotonic acid-citrate-dextrose, pH 6·5 as anticoagulant and PRP was prepared by centrifugation. The PRP was incubated at 37 °C for 15 min and then mixed with an equal volume of ACD. It was then centrifuged at 1000*g* for 15 min and the supernatant removed. The pellet was resuspended in a volume of HEPES-buffered saline (NaCl 145 mM, KCl 5mM, $MgSO_4$ 1 mM, glucose 10 mM, 4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid 10 mM, pH 7·4) sufficient to give a platelet concentration of about 5×10^8 mL^{-1}.

Platelet aggregation and release reaction

Platelet aggregation was monitored by following the changes in light absorbance that occurred after adding an aggregating agent to PRP or a suspension of washed platelets stirred at 37 °C. The platelet release reaction was quantified by measuring the amount of [^{14}C]5-HT that had been released from labelled platelets in response to the aggregating agent (Heptinstall & Fox 1983). The amount of [^{14}C]5-HT released was expressed as a percentage of the amount taken up into the platelets during the labelling procedure. Platelet aggregating agents that were used were arachidonic acid (AA), adrenaline, collagen and phorbol 12-myristate 13-acetate (PMA).

Quantification of the number of acid-soluble and acid-insoluble solphydryl groups in platelets

Samples of PRP were incubated with feverfew extract or parthenolide and then the number of acid-soluble and acid-insoluble sulphydryl groups in the platelets were determined using the method of Beutler et al (1963) as modified by Hofmann et al (1983). The method involves the use of dithiobis-*p*-nitrobenzoic acid which forms a complex with sulphydryl groups which absorbs at 405 nm.

Protein electrophoresis

Platelets (in PRP) that had been treated in various ways were washed, resuspended in buffered saline (pH 7·4) and then solubilized using 1% (w/v) sodium dodecyl sulphate (SDS) as described by Ostermann et al (1982). Sulphydryl groups were protected from oxidation by 10 mM *N*-ethylmaleimide. Polyacylamide gel electrophoresis (PAGE) was performed in 0·3% agarose–3·5% acrylamide gels according to Liu et al (1977).

Analysis of platelet suspensions for [^{14}C]AA metabolites

Samples (0·25 mL) of the platelet suspensions that had been incubated with [^{14}C]AA were acidified by adding 25 μL of 2 M formic acid and then extracted three times with ethyl acetate (0·5 mL). The combined extracts were washed twice with H_2O (0·5 mL) and then dried by adding a small amount of anhydrous $MgSO_4$. The extracts were then dried under N_2, redissolved in ethyl acetate (50 μL) and transferred to silica gel thin layer chromatography (TLC) plates (Kavalier, Czechoslovakia). The plates were developed using the upper phase of ethyl-acetate–iso-octane–acetic acid–H_2O (11 : 5 : 2 : 10) (Bryant & Bailey 1980a). After drying, the plates were sprayed with a solution of potassium iodide to locate peroxide-positive material (Oette 1965) and then 0·5 cm sections of the lanes of the plates were transferred to vials for liquid scintillation counting.

RESULTS

The effect of feverfew extract on the platelet aggregation and release reaction induced by some aggregating agents

To confirm that the feverfew extract exerted an inhibitory effect on aggregation and 5-HT release, aliquots (100 μL) of the extract (or PBS as control) were added to PRP (460 μL) and the extent of the aggregation and release induced by the subsequent addition of a solution (40 μL) of arachidonic acid (AA, final concentration 1 mM) or adrenaline (final

concentration 180 μM) was determined. The aggregating agent was added to some samples of PRP immediately following the addition of feverfew extract and added to others after the PRP had been incubated with extract at 37 °C for 15 min. The aggregation and release that occurred are shown in Fig. 1. It can be seen that the feverfew extract inhibited the aggregation and release induced by both aggregating agents and that greater inhibition was achieved after pre-incubation with the extract.

In other experiments samples of platelets that had been removed from plasma and resuspended in HEPES-buffered saline were incubated with feverfew extract and collagen or AA was then added. The extract inhibited the platelet aggregation and release of [^{14}C]5-HT induced by collagen and AA at concentrations similar to those needed to inhibit aggregation and release in PRP (data not shown).

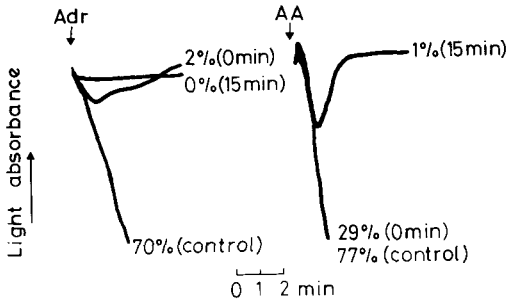

FIG. 1. Effects of feverfew extract on platelet aggregation and 5-HT release. Feverfew extract or saline (as control) was added to PRP and the extent of aggregation and 5-HT release that occurred in response to adrenaline (Adr) or AA was determined. Adrenaline or AA was added immediately (0 min) after adding the extract to the PRP or after incubating the PRP with extract for 15 min. The amounts of 5-HT (%) that were released from the platelets are shown at the end of the aggregation traces.

The effect of reagents containing sulphydryl groups on platelet aggregation and release in the presence of feverfew extract

An experiment was performed to determine the effect of reagents containing sulphydryl groups on platelet aggregation and release of [^{14}C]5-HT in the presence of feverfew extract. Samples of PRP (460 μL) were incubated with feverfew extract (100 μL) or cysteine (25 μL, final concentration 1 mM) or both feverfew and cysteine at 37 °C for 2 min. Adrenaline (24 μL, final concentration 100 μM) was then added and the effects of the various additions on aggregation and release were determined. The results are given in Fig. 2. Fig. 2 also contains the results of adding *N*-(2-mercaptopropionyl)glycine (2-MPG, 10 μL, final concentration 1·25 mM) in place of cysteine. It can be seen that both of these sulphydryl agents protected the platelets against inhibition of aggregation and release by feverfew. Similar results (not shown) were obtained when arachidonic acid was used in place of adrenaline. Neither glycine, serine, lysine, ornithine nor histidine (all at a concentration of 1 mM) used in place of cysteine or 2-MPG protected the platelets against inhibition of aggregation and release by feverfew extract.

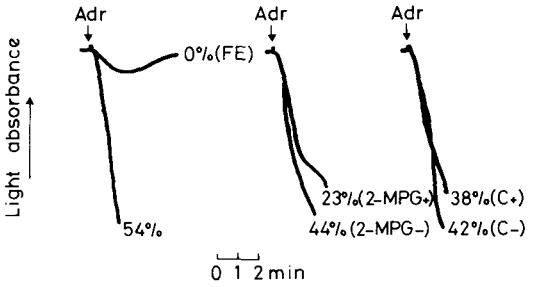

FIG. 2. Effects of 2-MPG and cysteine (C) on platelet aggregation and 5-HT release induced by adrenaline in the absence (−) and presence (+) of feverfew extract (FE). The various additions and the amounts of 5-HT (%) that were released from the platelets are shown at the end of the aggregation traces.

The effect of feverfew extract on the number of acid-soluble and acid-insoluble sulphydryl groups in platelets

Samples of PRP (500 μL) were incubated with feverfew extract (25, 50 or 100 μL) for different lengths of time and the number of acid-soluble and acid-insoluble sulphydryl groups were then determined. Feverfew induced a dose- and time-dependent fall in the number of acid-soluble sulphydryl groups in platelets (Fig. 3). There was also a small but inconsistent reduction in the number of acid-insoluble sulphydryl groups (data not shown).

In other experiments, we incubated samples of PRP with various amounts of feverfew extract or parthenolide (for 30 min) and then determined the number of acid-soluble and acid-insoluble sulphydryl groups present. It can be seen that adding parthenolide led to a dramatic reduction in the number of acid-soluble sulphydryl groups (Fig. 4a) and, again, there was a small effect on acid-insoluble sulphydryl groups. Very similar results to those obtained using parthenolide were obtained using feverfew extract (data not shown).

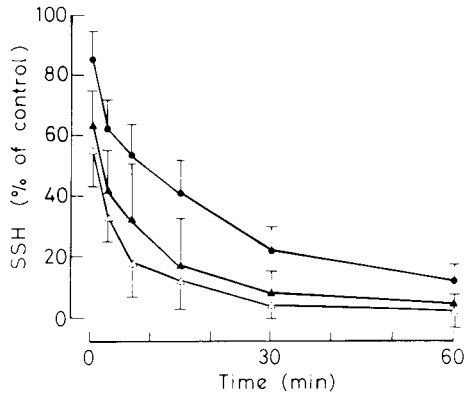

FIG. 3. Effects of different amounts of feverfew extract (FE) on the number of acid-soluble sulphydryl (SSH) groups in platelets. Samples of PRP were incubated with FE for different lengths of time and the results (mean ± s.d. n = 3) are expressed as a percentage of the number of SSH groups before incubation. The amounts of FE used were 25 μL (●), 50 μL (▲) and 100 μL/500 μL PRP (△).

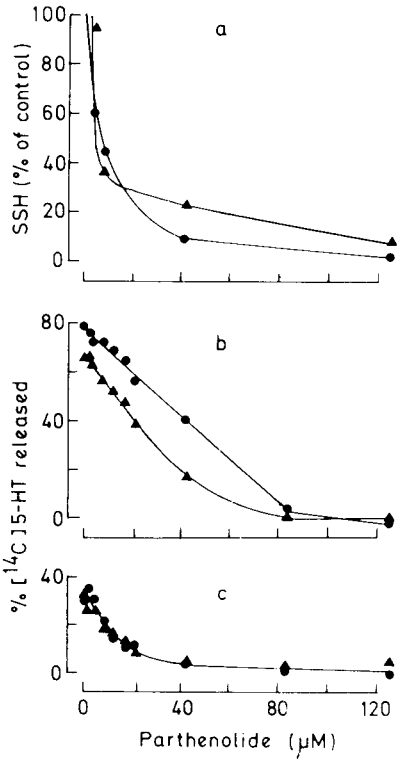

FIG. 4. Effects of different concentrations of parthenolide on (a) the number of acid-soluble sulphydryl (SSH) groups in platelets, and the amount (%) of [^{14}C]5-HT released from platelets in response to (b) AA and (c) PMA. In each case data from two experiments using different samples of PRP are shown.

We also determined the effects of feverfew extract and parthenolide on the ability of arachidonic acid (final concentration 1 mM) and PMA (0·8 μM) to induce a release reaction in samples of PRP. The experiments were carried out in exactly the same way as those to determine the effects of feverfew extract and parthenolide on the number of sulphydryl groups present. The results obtained for parthenolide are shown in Fig. 4b, c. It can be seen that secretion induced by PMA is more sensitive to inhibition by parthenolide than is the secretion induced by AA. Nevertheless, the amounts of parthenolide needed to inhibit secretion were similar to the amounts needed to reduce the number of sulphydryl groups in platelets. Again, very similar results to those obtained using parthenolide were obtained using feverfew extract (data not shown).

The effect of cysteine on the number of acid-soluble sulphydryl groups in the presence of feverfew extract

Since cysteine (but not glycine, serine or several other amino acids) can protect platelets against the inhibitory effects of feverfew extract on platelet aggregation and release of [^{14}C]5-HT, the influence of cysteine, glycine and serine on the number of soluble sulphydryl groups in platelets in the presence of feverfew extract was determined. The amino acids were added to feverfew extract or PBS (at a concentration of 2 mM) and the solutions were incubated at 37 °C for 5 min. Aliquots (100 μL) were then added to PRP (1 mL) and the samples incubated at 37 °C for 30 min. The number of acid-soluble sulphydryl groups in the platelets were then determined. In a parallel experiment in which samples of PRP were treated in exactly the same way the effects of the agents on the platelet aggregation induced by adrenaline (final concentration 180 μM) were determined. It can be seen (Table 1) that feverfew extract reduced the number of acid-soluble sulphydryl

Table 1. Effect of cysteine in comparison with glycine and serine on the number of acid-soluble sulphydryl (SSH) groups in platelets in the absence and presence of feverfew extract (FE).

	SSH	
	−FE	+FE
No addition	100	20
Cysteine	109	82
Glycine	112	20
Serine	100	23

Results are expressed as % of control.

groups to 20% of the control, that cysteine protected the platelets against this loss, but that glycine and serine were ineffective. Similarly feverfew inhibited platelet aggregation, and cysteine (but not glycine or serine) protected the platelets from this inhibitory effect (data not shown).

The effect of feverfew extract on platelet proteins

Samples of PRP (500 μL) were incubated with feverfew extract (25 to 100 μL for 15 min), the platelets were then washed and solubilized and the products subjected to gel electrophoresis. In a parallel experiment a sample of PRP was incubated with diamide (azodicarboxylic acid- bis-dimethyl-amide, 0·5 mM for 30 min). Diamide is an agent that oxidizes sulphydryl groups (Kosower et al 1969) and causes formation of disulphide-linked polymers of platelet proteins (Bosia et al 1983; Spangenberg et al 1984). The results of our experiments using feverfew and diamide are shown in Fig. 5. As expected, incubation with diamide led to a reduction in the amount of actin-binding protein (ABP), P230 and myosin heavy chain (MYO) with formation of new high molecular weight proteins P_a and P_c as previously described by Spangenberg et al (1984). In contrast, after incubation with feverfew extract the gel electrophoretogram did not differ from the control.

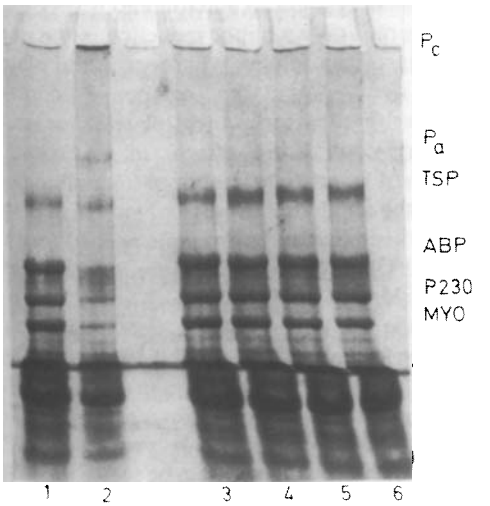

FIG. 5. Patterns of platelet proteins obtained by SDS-PAGE after treating PRP in different ways: 1 untreated PRP, 2 treatment with diamide (0·5 mM), 3–6 treatment with feverfew extract (25, 50, 75 and 100 μL/500 μL PRP). The molecular weights of the various proteins are: MYO (200 000), P230 (230 000), ABP (260 000), TSP (360 000), P_a (ca. 400 000), P_c (>1 million). See text for explanation of abbreviations.

However, feverfew did produce changes in the protein pattern after the platelets had been treated subsequently with aggregating agents (Fig. 6). Adrenaline (final concentration 180 μM) or AA (final concentration 1 mM) was added to PRP and platelet aggregation allowed to proceed for 5 min before the reaction was terminated for analysis of platelet proteins. To other samples of PRP, feverfew extract (100 μL) was added and the PRP incubated for 15 min before stimulation with adrenaline or AA (for 5 min). After aggregation of the platelets with AA or adrenaline alone the only notable change was a decrease in the amount of thrombospondin (TSP) in the platelets. Preincubation with extract led to formation of some P_a and prevented release of thrombospondin from the platelets. The presence of disulphide bonds in the P_a was confirmed by reducing the product with dithiothreitol before protein analysis. After reduction the P_a was absent.

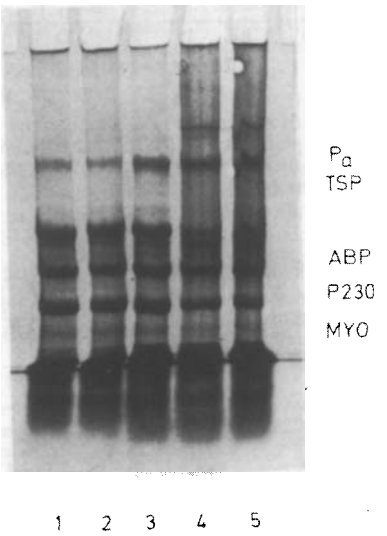

FIG. 6. Patterns of platelet proteins obtained by SDS-PAGE after treatment of PRP in different ways: 1 treatment with adrenaline, 2 treatment with AA, 3 untreated PRP, 4 treatment with adrenaline after addition of feverfew extract, 5 treatment with AA after addition of feverfew extract. See text for explanation of abbreviations.

The effect of feverfew extract on the metabolism of exogenous arachidonic acid by platelets

A sample of washed platelets in HEPES-buffered saline (480 μL) was incubated with feverfew extract (120 μL) at 37 °C for 15 min; a control sample contained PBS in place of feverfew extract. The samples were then transferred to an aggregometer, stirred, a solution of $CaCl_2$ (final concentration

1 mM) was added, and this was followed by [^{14}C]AA (5 μL, final concentration 10 μM). The light absorbance of the control sample fell rapidly as the platelets aggregated but the light absorbance of the sample that contained feverfew indicated only a rapid, transient reversible aggregation response. After 5 min stirring the samples were analysed for [^{14}C]AA metabolites by TLC. The results are given in Fig. 7 and Table 2. Table 2 also contains the results of a second identical experiment.

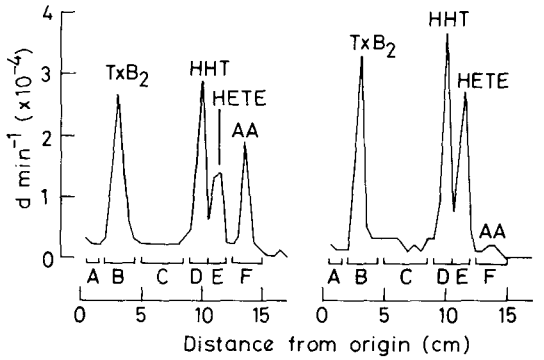

FIG. 7. Patterns of AA metabolites obtained by TLC after treating washed platelets with [^{14}C]AA in the absence (left) and presence (right) of feverfew extract. Various regions of the TLC plates are designated A–F.

Table 2. Effects of feverfew extract (FE) on [^{14}C]AA metabolism in washed platelets. Amounts of radioactivity (% of total radioactivity recovered from the plate) in different regions of the TLC plate (see Fig. 7). Results of two separate experiments are given.

		AA		FE + AA	
Metabolite	Region	Exp. 1	Exp. 2	Exp. 1	Exp. 2
	A	4	2	2	2
TXB_2	B	32	20	31	19
	C	8	4	10	12
HHT	D	24	20	27	29
HETE	E	15	16	26	23
AA	F	16	37	4	14

It can be seen that the levels of thromboxane B_2 (TXB_2, region B in the chromatogram shown in Fig. 7) did not change with pre-incubation with feverfew extract, but that the extract brought about increased conversion of AA (region F) to material in region C, 12-hydroxyheptadecatrienoic acid (HHT, region D) and 12-hydroxyeicosatetraenoic acid (HETE, region E). Material in region C is thought to include trihydroxyeicosatrienoic acid (THETE) and hydroxy-epoxyeicosatrienoic acid (HEPA), Bryant & Bailey 1980a,b). After treatment with feverfew extract and AA (but not with AA alone) peroxide-positive material was observed to co-migrate with HETE, indicating incomplete conversion of 12-hydroperoxy-eicosatetraenoic acid (HPETE) to HETE in the presence of feverfew extract (Loesche et al 1984).

DISCUSSION

The structures of the active components of feverfew suggest that their effects on platelet behaviour may be via interaction with sulphydryl groups in or on the platelets (Groenewegen et al 1986). The results of the present investigation are not incompatible with such a mode of action.

First, the inhibitory effects of feverfew extract are prevented by prior treatment with compounds such as cysteine or 2-MPG which contain sulphydryl groups. Presumably this is through formation of inactive Michael addition products via the α-methylene-butyrolactone unit of the sesquiterpene lactones in the extract. Several other amino acids that we tested which contained nucleophilic groups other than sulphydryl groups (hydroxy and amino groups) did not prevent the inhibitory effects of feverfew on platelet behaviour.

Second, both feverfew extract and parthenolide (one of the sesquiterpene lactones present in feverfew) reduce the number of sulphydryl groups in platelets. Their effect on the acid-soluble sulphydryl groups (mainly reduced glutathione) is very marked while there is only a minor reduction in the number of acid-insoluble sulphydryl groups in the platelets. Again, it was found that compounds containing sulphydryl groups can protect platelets against the sulphydryl-neutralizing effects of feverfew. The amounts of feverfew extract or parthenolide needed to alter the number of sulphydryl groups in platelets are not dissimilar to the amounts needed for inhibition of [^{14}C]5-HT release in response to arachidonic acid or PMA.

Diamide is an agent that encourages protein polymer formation via oxidation of sulphydryl groups contained within them to form disulphide linkages (Bosia et al 1983; Spangenberg et al 1984). The effect of diamide on platelet proteins is shown in Fig. 5. In the same Figure it can be seen that feverfew extract does not induce protein polymer formation directly. However, protein polymer formation does occur when platelets are preincubated with feverfew extract and then treated with a platelet aggregating agent. It may be concluded that lowering the amount of reduced glutathione in platelets is insufficient to produce disulphide linkages in proteins. However, during platelet aggregation oxidative species are

generated (Marnett et al 1985) and in the absence of sufficient glutathione to scavenge such species, oxidation of protein sulphydryl groups could occur.

Finally incubation of platelets with feverfew extract lead to a change in [^{14}C]AA metabolism in washed platelets. The rate of metabolism was increased with increased production of products of the lipoxygenase pathway. In addition, there was evidence of impaired conversion of the primary lipoxygenase product HPETE to HETE. These are typical features of metabolism in glutathione-depleted platelets (Bryant & Bailey 1980b; Bryant et al 1983; Loesche et al 1984).

Despite the marked effect of feverfew extract and parthenolide on the number of acid-soluble sulphydryl groups (reduced glutathione) in platelets, it is unlikely that this is the explanation of their inhibitory effects on platelet behaviour. Iodoacetamide also alkylates soluble sulphdryl groups in platelets but has much less effect on platelet behaviour than feverfew extract or parthenolide (Bosia et al 1983). 1-Chloro-2,4-dinitrobenzene is another agent that removes reduced glutathione. However, unlike feverfew, it is reported to have little effect on platelet behaviour in PRP (Bosia et al 1985).

Although there are only minor changes in the number of acid-insoluble (protein) sulphydryl groups in platelets, it is possible that the inhibitory effects of feverfew extract on platelet behaviour are via neutralization of a small number of sulphydryl groups on specific enzymes of proteins that are fundamental to platelet aggregation and secretion. It is interesting that the secretion induced by PMA is more sensitive to inhibition by feverfew extract and parthenolide than secretion induced by AA. Perhaps greater numbers of sulphydryl groups are required for PMA to exert its secretory effect.

Clearly attempts should now be made to identify the particular enzymes or proteins that are the target of the sesquiterpene lactones present in feverfew. Their identification would provide more insight into the mechanisms underlying platelet aggregation and secretion and may provide a rational pharmacological approach to inhibition of platelet behaviour.

Acknowledgements

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