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Toxic inhibition of smooth muscle contractility by plant-derived sesquiterpenes caused by their chemically reactive α-methylenebutyrolactone functions

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1 Previous studies have shown that extracts of feverfew (*Tanacetum parthenium*) and parthenolide, a sesquiterpene α-methylenebutyrolactone obtained from it, inhibit smooth muscle contractility in a time-dependent, non-specific and irreversible manner.

2 The hypothesis that this toxic effect is due specifically to the presence in the sesquiterpene lactone of the potentially reactive α-methylene function was tested on rabbit isolated aortic ring preparations. This was done (a) by comparing the effects of two plant-derived sesquiterpene lactones purified from yellow star thistle (*Centaurea solstitialis*): cynaropicrin (an α-methylenebutyrolactone) and solstitialin 13-acetate (lacking the α-methylene function), and (b) by chemically inactivating the α-methylene functions in cynaropicrin and parthenolide by reaction with cysteine.

3 The results show that the characteristic smooth muscle inhibitory profile is demonstrated by the two α-methylenebutyrolactones (parthenolide and cynaropicrin), but not by the compound lacking this functional group (solstitialin 13-acetate), or by those previously active compounds in which it has been inactivated with cysteine.

4 Thus the α-methylene function is critical for this aspect of the toxic pharmacological profile of the sesquiterpene butyrolactones, which are natural products widely distributed in the Compositae family of flowering plants.

Keywords: Smooth muscle; irreversible antagonism; natural products; sesquiterpene lactones; feverfew; yellow star thistle; Compositae/Asteraceae; sulphydryl groups

Introduction

Feverfew (*Tanacetum parthenium*, Compositae, alternatively known as Asteraceae) is a garden plant which is widely used in the UK for self-medication of arthritis and migraine but the pharmacological basis for its claimed effects is not known (Berry, 1984; Editorial, Lancet 1985). Recent studies showed that extracts of fresh leaves contain sesquiterpene α-methylenebutyrolactones such as parthenolide which cause irreversible, time-dependent and non-specific inhibition of aortic smooth muscle contractility (Barsby *et al.*, 1992; 1993). In contrast, extracts of dried powdered leaves available from health food shops do not inhibit aortic contractility and do not contain α-methylenebutyrolactones (Barsby *et al.*, 1993).

In order to understand the molecular mechanisms underlying the inhibition of smooth muscle contractility, it is necessary to establish which chemical groups in the sesquiterpene α-methylenebutyrolactones are responsible. The exocyclic α-methylene function is capable of reacting with thiols such as cysteine residues of amino acids, thereby forming Michael adducts (Rodriguez *et al.*, 1976; Berry, 1984; Groenewegen *et al.*, 1986). The hypothesis that this may be responsible for the toxic actions of certain sesquiterpene lactones can be tested by comparing compounds of closely similar structure but which differ in the presence or absence of α-methylenebutyrolactone functional groups.

We have therefore studied the effects on rabbit aortic rings of two other plant-derived sesquiterpene lactones which have been purified from yellow star thistle (*Centaurea solstitialis*): cynaropicrin (an α-methylenebutyrolactone) and solstitialin 13-acetate (lacking the α-methylene function). We have also investigated the consequences of chemically inactivating the α-methylene functions in cynaropicrin and parthenolide by reaction with cysteine. Yellow star thistle is a member of a genus known to be especially rich in sesquiterpene lactones, and many have now been identified (Rodriguez *et al.*, 1976; Herz, 1977), some of which may cause the toxicity of various species of Compositae to livestock (Rodriguez *et al.*, 1976).

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Methods

Rings of 2 mm thickness were carefully cut from the thoracic segment of the aorta taken from male New Zealand White rabbits (2.0–3.0 kg) and suspended for isometric recording under a load of 2 g in 3 ml tissue baths containing well-oxygenated Krebs solution as described earlier (Barsby *et al.*, 1992). Contractions were induced by the addition of 10^{-6} M phenylephrine or 40 mM KCl.

Parthenolide was isolated from *T. parthenium* and characterized as described (Dolman *et al.*, 1992), and was dissolved in methanol for biological testing. Cynaropicrin and solstitialin 13-acetate were isolated from *C. solstitialis* and characterized as reported previously (Wang *et al.*, 1991); they were dissolved in dimethylsulphoxide (DMSO) for biological testing. As before, these compounds were characterized by ^{1}H and ^{13}C nuclear magnetic resonance (n.m.r.) and the purity was greater than 97% by thin layer chromatography (t.l.c.).

The structures of the three sesquiterpene lactones are shown in Figure 1. For some experiments, parthenolide and cynaropicrin were dissolved at 25 mg ml^{-1} in methanol or DMSO containing 100 mg ml^{-1} cysteine and allowed to react overnight before testing. Parallel stocks were prepared without cysteine. To establish whether indeed satisfactory addition of cysteine to the α-methylene function had occurred, chemical high performance liquid chromatography (h.p.l.c.) analysis of the product of the reaction with parthenolide was performed (amounts of cynaropicrin-cysteine were insufficient). This was done using the procedure described by

Dolman et al. (1992) in which the adducts formed from the reaction of 5–10 mg samples of the parthenolide or parthenolide-cysteine with excess 9-thiomethylanthracene in chloroform are quantified using a 10 μm μPorasil column eluted at 3 ml min^{-1} with 55% chloroform–45% hexane and the detector set at 0.08 sensitivity, 369 nm.

Results

Representative records of the responses of the aortic rings to application of phenylephrine are shown in Figure 2. Addition of parthenolide or cynaropicrin at a final bath concentration of 100 μg ml^{-1} caused a slow and prolonged decline in tension of the rings, whereas addition of solstitialin 13-acetate or the equivalent volumes of the vehicles (methanol or DMSO) did not (Figure 2). After repeated washout of the drugs and an extended recovery period of 30–90 min, further tests with phenylephrine were made. No responses were obtained in tissues treated with parthenolide or cynaropicrin, whereas full responsiveness was retained after the other treatments (Figure 2). Similar results were obtained using potassium chloride to induce contractions, although traces are not shown here.

Cynaropicrin and parthenolide were treated with cysteine as described in Methods. Addition of these chemically modified sesquiterpene lactones to aortic rings did not have

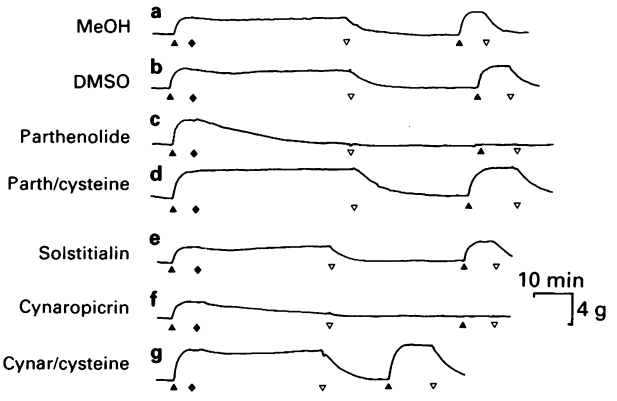

Figure 2 Effects of sesquiterpene lactones on the contractile function of rabbit aortic rings. Panels (a) to (g) show the effects of the addition of (a) methanol, (b) dimethylsulphoxide, (c) parthenolide, (d) parthenolide reacted with cysteine, (e) solstitialin 13-acetate, (f) cynaropicrin, (g) cynaropicrin reacted with cysteine. Addition of 10^{-6} M phenylephrine was at ▲ and washout was at ▽. The lactones or vehicle were added once at ◆, after plateau contractions were attained. Similar results were obtained in tests on *n* other rings, as shown in Table 1.

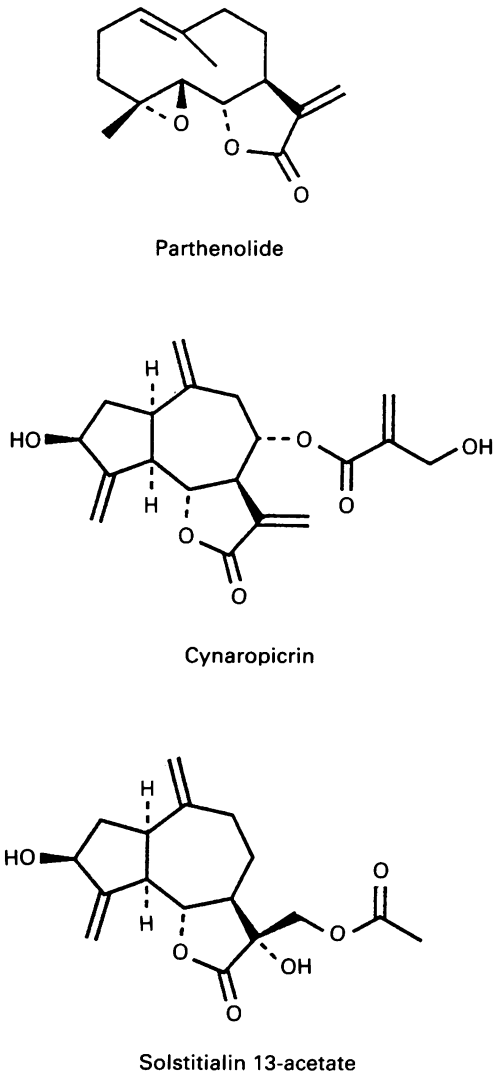

Figure 1 Structures of the three sesquiterpene lactones.

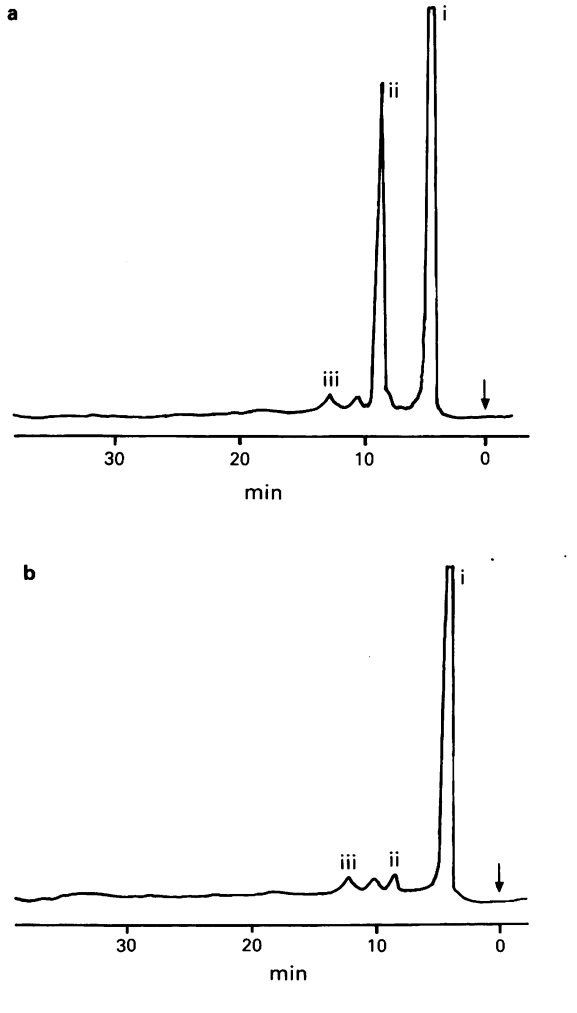

Figure 3 Elution profiles from chemical-h.p.l.c. assay of parthenolide before and after reaction with cysteine. Panel (a) shows parthenolide, (b) is from a sample of parthenolide after treating with cysteine. Peak (i) is excess 9-thiomethylanthracene, peak (ii) is the parthenolide 9-thiomethylanthracene adduct, peak (iii) is a reagent impurity. Full scale deflection is 0.08 absorbance units at 369 nm.

Table 1 Inhibition of contractility of rabbit aortic rings by sesquiterpene lactones: effect of lack of α-methylenebutyrolactone functions or their inactivation by cysteine

Test substance†	*Loss of tone in rings precontracted with phenylephrine (mg min^{-1})*	*% inhibition of subsequently applied phenylephrine*	*Number of tests*
Methanol vehicle	4.9 ± 2.5	− 3.2 ± 5.7	5
Methanol + cysteine	0.9 ± 0.9	− 1.7 ± 6.1	3
DMSO vehicle	10.5 ± 3.8	− 5.8 ± 3.7	7
DMSO + cysteine	24.8 ± 6.7	3.2 ± 3.9	4
Parthenolide	132.7 ± 20.7*	98.2 ± 1.3*	6
Parthenolide + cysteine	14.0 ± 5.3	4.0 ± 4.2	6
Cynaropicrin	84.7 ± 8.0*	94.8 ± 2.0*	9
Cynaropicrin + cysteine	7.3 ± 3.1	− 4.0 ± 2.7	7
Solstitialin 13-acetate	9.1 ± 4.6	− 9.2 ± 4.0	6

†Rings were set up in 3 ml baths as described and contractions to phenylephrine and KCl obtained until reproducible. The rings were then contracted with 10^{-6} M phenylephrine. Then 5 min later (at which time the response had reached a plateau) 12 μl of vehicle or test substance (final concentration 100 μg ml^{-1}) was added and allowed to remain in contact with the phenylephrine-contracted tissue for 30 min. The rate of change of tension of the rings (loss of tone) is shown in column 2. The preparations were then washed thoroughly to remove agonist and inhibitor, and allowed to rest for 30–90 min. The agonist was then retested, and the magnitude of the subsequent response related to the previous value (shown in column 3). Values are means ± s.e.mean for tests on the numbers of different ring preparations shown in column 4. *Indicates statistically significant difference from corresponding vehicle control and from corresponding cysteine treatment by Student's unpaired t test, $P<0.01$.

any effect on agonist-induced tone, and did not alter subsequent agonist responsiveness after washout (Figure 2). Thus reaction with cysteine abolished the inhibitory activity of these two lactones on the aortic rings. Chemical-h.p.l.c. assay was used in the case of the parthenolide-cysteine adduct to check that the reactive α-methylene function had been removed. Figure 3 shows that the characteristic peak due to the presence of an adduct formed by reaction of 9-thiomethylanthracene with the free α-methylene function of parthenolide is no longer found if the parthenolide had been treated with cysteine.

The combined data for this series of experiments are collected in Table 1.

Discussion

These experiments provide strong evidence that the smooth muscle inhibitory effects of parthenolide and other sesquiterpene α-methylenebutyrolactones are due to the chemically reactive α-methylenebutyrolactone function. Its neutralization with cysteine abolishes inhibitory activity. Moreover, solstitialin 13-acetate, which does not contain the α-methylene function is not effective, whereas the closely related lactone, cynaropicrin, is fully inhibitory.

Clearly, these plant-derived substances are toxic to smooth muscle, because their application at 100 μg ml^{-1} (about 400 μM) produces irreversible loss of tone and subsequent inability to contract to agonists. We have found that washing the rings for up to 15 h does not lead to any return of contractility (unpublished experiments). This fact, together with the slow onset of inhibitory action and the known capacity of α-methylenebutyrolactones to react with sulphydryl groups, suggests that this toxic pharmacological effect is due to covalent modification of some as yet unidentified protein which is essential to the smooth muscle contractile apparatus.

The chemical reactivity of α-methylenebutyrolactones has been invoked as the explanation for other effects of these very widely distributed natural products (Rodriguez *et al.*, 1976; Berry, 1984). For example, they demonstrate potent anti-inflammatory actions in rodents (Hall *et al.*, 1980), are cytotoxic (Rodriguez *et al.*, 1976; Berry, 1984), cause allergic contact dermatitis (Rodriguez *et al.*, 1976; Berry, 1984) and inhibit secretion from platelets (Groenewegen *et al.*, 1986). However, it is not known whether the sesquiterpene lactones are responsible for the claimed therapeutic benefits of the feverfew plant, although they may be responsible for some of its side effects (Berry, 1984). There are also indications from *in vitro* neurotoxicity studies (Wang *et al.*, 1991; Cheng *et al.*, 1992) that α-methylenebutyrolactones might be responsible for the central neurotoxic effects observed in equine nigropallidal encephalomalacia. This disease occurs in horses grazing on meadows overgrown with yellow star thistle (Cordy, 1978).

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