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Effect of triterpenoids on the inflammation induced by protein kinase C activators, neuronally acting irritants and other agents

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Abstract

In order to establish the mode of the anti-inflammatory activity of triterpenoids, 11 naturally occurring compounds were assayed on mouse ear oedema induced by the protein kinase C activators, mezerein, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), two 12-deoxyphorbol-13-monoesters (13-tetradecanoate (DPT) and 13-phenylacetate (DPP)) and bryostatin 1, and by resiniferatoxin, xylene and arachidonic acid. The effects on bradykinin-induced paw oedema and on the rat skin inflammation caused by hydrogen peroxide were also examined. The oedema induced by mezerein and DPT was reduced to different extents by the triterpenoids administered epicutaneously (0.5 mg per ear). Against DPT-induced oedema, lupane and oleanane derivatives were the most effective compounds. Oleananes and lupanes possessing a carboxyl group were active against bryostatin 1-induced oedema. Most of the triterpenoids were ineffective against the neurogenic inflammation caused by resiniferatoxin and xylene. Many triterpenoids, especially oleanane and lupane alcoholic derivatives, were active against the plantar oedema induced by bradykinin and on the intradermal inflammation induced by hydrogen peroxide. In conclusion, the anti-inflammatory activity of triterpenoids may depend on inhibition of protein kinase C, without any involvement of neurogenic inflammatory mechanisms.

Keywords: Bradykinin; Inflammation; Neurogenic; Phorbol ester; Protein kinase C; Triterpenoid

1. Introduction

Triterpenoids have been described as effective anti-inflammatory agents and are recognised as the active principles of several therapeutically used medicinal plants such as liquorice, ginseng or horse chestnut. A large number of triterpenoids have now been reported to act as anti-inflammatories through various mechanisms, including inhibition of eicosanoid and cytokine production, hydrolytic enzyme activity, lipid peroxidation (Safayhi and Sailer, 1997), and interaction with some serine/threonine kinases (Hasmeda et al., 1999). One of the most important enzymes of this kind is protein kinase C, a calcium/phospholipid-dependent enzyme that regulates vast numbers of physiological events such as cellular differentiation and proliferation. It consists of at least 12 distinct isotypes described on the basis of their substrate, cofactor requirements, tissue expression, kinetics of membrane association and protein sequence (Dekker and Parker, 1994; Mellor and Parker, 1998). Soon after the classical forms of protein kinase C were discovered, the diterpenoid, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), was identified as one of its most powerful activators, because it is a stable, lipophilic analogue of diacylglycerol (Castagna et al., 1982; Nishizuka, 1984).

Many constituents of the resins naturally found in various organs of some Euphorbiaceae and Thymeleaceae plants belong, like TPA, to the tigliane class of diterpenoids and are termed *phorbol esters* because they contain acyl moieties substituting one or more hydroxyl functions on the phorbol structure. Most of these compounds, together with some related daphnane and ingenane esters, have co-carcinogenic, skin irritant and profound inflamma-

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tory properties that have been extensively studied (Hergenhahn et al., 1974; Alcaraz and Ríos, 1991). Among these compounds, the inflammatory effect seems to be even more widespread than the co-carcinogenic one. In fact, several 12-deoxyphorbol esters are potent inflammatory agents but have no effect as tumour promoters (Driedger and Blumberg, 1980).

In addition to the multiple relationships between diterpenoids, inflammation and protein kinase C activity, there is some evidence that some of these compounds, especially resiniferatoxin, are neurogenic inflammatory agents. Resiniferatoxin is a daphnane isolated from *Euphorbia resinifera* (Hergenhahn et al., 1975) and later characterised as an ultra-potent analogue of capsaicin that interacts with the vanilloid receptor on afferent C-fibers and mediates inflammation and pain (Appendino and Szallasi, 1997).

The present study continued in the line of our previous reports on triterpenoid structure–activity relationships (Recio et al., 1995a,b; Máñez et al., 1997) and examined the effects of 11 triterpenoids and several standard drugs against the mouse ear oedema induced by the protein kinase C activators, TPA, 12-deoxyphorbol-13-tetradecanoate (DPT), 12-deoxyphorbol-13-phenylacetate (DPP), mezerein and bryostatin 1, and by the neurogenic inflammation elicitors, resiniferatoxin and xylene. The effects of these compounds against the ear oedema induced by arachidonic acid, against the foot-paw oedema induced by bradykinin, and against the rat skin inflammation induced by free reactive oxygen species were also studied. Use of this type of experiments allowed us to distinguish between the effect of triterpenoids against protein kinase C-mediated and that against neuronally driven inflammatory processes.

2. Material and methods

2.1. Animals

Groups of six female Swiss mice weighing 25–30 g, or six female Wistar rats weighing 200–250 g were used. All animals were fed a standard diet ad libitum. Housing conditions and experimental procedures were in accordance with the European Union regulations on the use of animals for scientific purposes (CEE Council 86/609).

2.2. Chemicals

Triterpenoids: betulinic acid, erythrodiol and hederagenin were purchased from Roth (Karlsruhe, Germany); oleanolic, 18α- and 18-β-glycyrrhetinic acids, betulin, lupeol and uvaol from Sigma (St. Louis, USA); α-amyrin from Apin Chemicals (Abingdon, UK). We obtained ursolic acid from *Helichrysum stoechas* Moench. (Asteraceae) (Recio et al., 1991). Inflammatory agents: mezerein, DPT, TPA, resiniferatoxin, arachidonic acid (99%), bradykinin and glucose oxidase type V-S from *Aspergillus niger* (200,000 units g^{-1}) in 0.1 M acetate buffer from Sigma; bryostatin 1 and DPP, from ICN Pharmaceuticals (Costa Mesa, USA), and xylene (analytical grade) from Panreac (Sta. Perpètua, Spain).

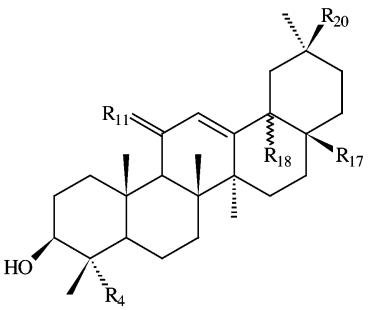
Standard drugs: carbamazepine, catalase-PEG 5000 (40,000 units mg^{-1}), dexamethasone, isoprenaline hemisulphate, indomethacin, [Leu^8]des-Arg^9-bradykinin acetate, lidocaine, nordihydroguaiaretic acid, phenidone and trifluoperazine dihydrochloride from Sigma; capsazepine from ICN Pharmaceuticals; and GF-109203X (3-[1-[3-(dimethylamino)propyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione) from RBI (Natick, USA).

Other products: acetone (analytical grade) from Merck (Darmstadt, Germany); ethanol 96% (analytical grade) and polyoxyethylenesorbitan monooleate (tween 80) from Panreac; and ketamine hydrochloride and methoxypolyethylene glycol succinimidyl succinate (PEG 5000) from Sigma.

2.3. Mouse ear oedema induced by various agents: mezerein, DPT, TPA, DPP, bryostatin 1, resiniferatoxin, xylene and arachidonic acid

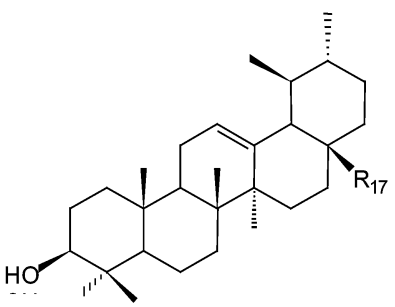
Oedema was induced on the right ear by topical application of the irritant dissolved in 20 μl acetone (10 μl + 10 μl, on both surfaces), at the specified dose: 6.5 μg per ear mezerein (Szallasi and Blumberg, 1989), 1 μg per ear DPT, 2.5 μg per ear TPA (Payá et al., 1993), 4 μg per ear DPP, 0.89 μg (1 nmol) per ear bryostatin 1 (Gschwendt et al., 1992), 10 μg per ear resiniferatoxin (Szallasi and Blumberg, 1989), 20 μl per ear xylene and 2 mg per ear arachidonic acid (Young and De Young, 1989). Test compounds (0.5 mg per ear), dissolved in 20 μl acetone or 80% aqueous ethanol, were applied on the same ear simultaneously, and 2 h or 30 min before the inflammatory agent. With the arachidonic acid-induced oedema, compounds were applied 30 min before arachidonic acid. Dexamethasone was applied at a dose of 0.05 mg per ear, always 2 h before the irritant. GF-109203X was administered at a dose of 0.05 mg per ear, simultaneously with bryostatin 1. In the studies of other standard drugs, these were administered according to two different regimes: simultaneously, and 30 min before the irritant. Capsazepine was given at a dose of 0.1 mg per ear in the resiniferatoxin-induced oedema. The thickness of the ears was measured using a Series 293 micrometer Mitutoyo, prior to the experiment and at the time stated after induction of inflammation: 4 h for mezerein, DPT and TPA, 16 h for bryostatin 1, 30 min for DPP, resiniferatoxin and xylene, and 1 h for arachidonic acid. Oedema was expressed as the increase in ear thickness due to the inflammatory challenge and oedema inhibition was expressed as the percent thickness reduction referred to the control

a) Triterpenoid structures

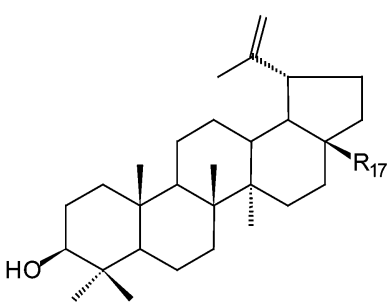


Oleanane triterpenoids

	R_4	R_{11}	R_{17}	R_{18}	R_{120}
Erythrodiol	CH_3	H_2	CH_2OH	H	CH_3
18 α-Glycyrrhetinic acid	CH_3	O	CH_3	αH	COOH
18 β- Glycyrrhetinic acid	CH_3	O	CH_3	βH	COOH
Hederagenin	CH_2OH	H_2	COOH	H	CH_3
Oleanolic acid	CH_3	H_2	COOH	H	CH_3



Ursane triterpenoids

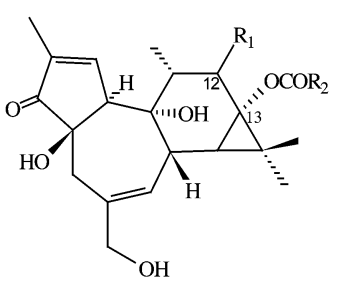


Lupane triterpenoids

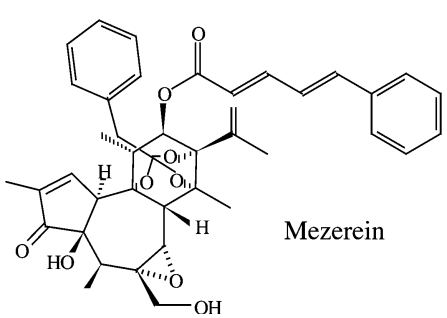
	R_{17}
α-Amyrin	CH_3
Uvaol	CH_2OH
Ursolic acid	COOH

	R_{17}
Lupeol	CH_3
Betulin	CH_2OH
Betulinic acid	COOH

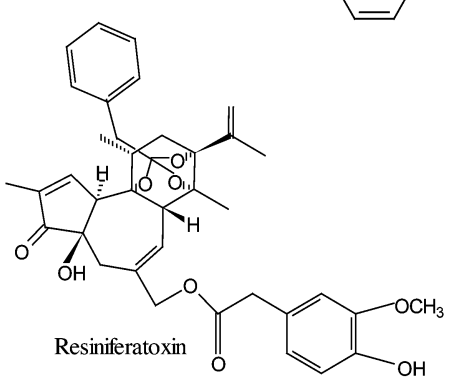
b) Irritant diterpenoids



	R_1	R_2
TPA	$OCO(CH_2)_{12}CH_3$	CH_3
DPT	H	$(CH_2)_{12}CH_3$
DPP	H	CH_2–



Mezerein



Resiniferatoxin

Fig. 1. (a) Structure of the triterpenoids tested. (b) Structure of the irritant diterpenoids, mezerein, resiniferatoxin, TPA, DPT and DPP.

Table 1
Effect of triterpenoids on mezerein-, 12-deoxyphorbol-13-tetradecanoate (DPT)- and 12-deoxyphorbol-13-phenylacetate (DPP)-induced mouse ear oedema

	Mezerein		DPT		DPP	
	ΔT (μm)	I%	ΔT (μm)	I%	ΔT (μm)	I%
Control	176.8 ± 13.7		107.1 ± 7.6		114.5 ± 17.1	
Betulin	98.0 ± 11.3[b]	45	49.2 ± 17.9[b]	54	75.1 ± 18.6[ns]	34
Betulinic acid	91.3 ± 6.2[b]	48	52.4 ± 13.8[b]	51	44.3 ± 14.2[b]	61
Lupeol	78.3 ± 10.4[b]	56	64.4 ± 8.5[a]	40	107.0 ± 15.7[ns]	7
Erythrodiol	59.0 ± 5.7[b]	67	57.8 ± 12.2[a]	46	73.9 ± 6.0[ns]	36
α-Glycyrrhetinic acid	173.1 ± 9.8[ns]	2	70.5 ± 8.2[ns]	34	71.7 ± 5.7[ns]	37
β-Glycyrrhetinic acid	110.1 ± 12.8[b]	38	62.5 ± 16.8[a]	42	73.3 ± 8.9[ns]	36
Hederagenin	195.8 ± 19.5[ns]	−11	52.2 ± 4.4[b]	56	78.1 ± 11.2[ns]	32
Oleanolic acid	229.4 ± 13.9[a]	−30	58.0 ± 15.5[a]	46	59.7 ± 19.1[a]	48
α-Amyrin	176.1 ± 12.0[ns]	0	71.7 ± 9.2[ns]	33	62.5 ± 12.7[ns]	45
Ursolic acid	67.3 ± 14.4[b]	62	74.7 ± 8.8[ns]	30	87.1 ± 9.0[ns]	24
Uvaol	218.2 ± 9.8[ns]	−23	63.7 ± 12.0[a]	41	86.4 ± 11.0[ns]	25
Indomethacin	66.4 ± 10.2[b]	62	48.1 ± 8.3[b]	55	62.0 ± 9.0[a]	46

Test compounds were applied topically (0.5 mg per ear) simultaneously with the irritants. Ear oedema was measured 4 h after irritant administration (mezerein and DPT) or 30 min after application of DPP. Values are expressed as means ± S.E.M for six animals (minimum). Statistical significance of difference from the control: [a]$P < 0.05$, [b]$P < 0.01$, [ns]$P > 0.05$. ΔT: Increase in ear thickness. I%: Inhibition percentage.

group. Each of the vehicles used as excipient for the test compounds had been shown to be pharmacologically inert (Payá et al., 1993).

2.4. Bradykinin-induced mouse paw oedema

The method was previously described by Tsurufuji et al. (1980). Briefly, 25 μl of a solution containing 3 μg bradykinin in saline was s.c. injected into the right paw, and 25 μl of saline into the left one. Triterpenoids (10 mg/kg) were dissolved in 0.1 ml of ethanol/tween 80/water (1:1:10), a mixture for which we had previously found no interference with the inflammation process, and were administered i.p. 30 min (1 or 3 h, as stated) before bradykinin. Isoprenaline was dissolved in 0.1 ml saline and administered i.p., 5 mg/kg, at the same time as the triterpenoids. Dexamethasone (1 mg/kg) was dissolved in 0.1 ml of ethanol/saline (1:19) and administered i.p. 3 h before bradykinin. The antagonist, [Leu[8]]des-Arg[9]-bradykinin, was conjointly administered with bradykinin at a dose of 10 μg. The volumes of both paws were measured with a plethysmometer (Ugo Basile) 12 min after the induction of swelling. Oedema was expressed as the difference between the right and left paw volume. Oedema

Table 2
Effect of triterpenoids as 2-h pretreatment on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-, mezerein- and 12-deoxyphorbol-13-tetradecanoate (DPT)-induced mouse ear oedema

	TPA		Mezerein		DPT	
	ΔT (μm)	I%	ΔT (μm)	I%	ΔT (μm)	I%
Control	188.0 ± 15.5		183.4 ± 12.3		107.7 ± 10.7	
Betulin	104.6 ± 11.8[b]	44	184.1 ± 13.8[ns]	0	59.4 ± 9.5[ns]	45
Betulinic acid	122.6 ± 30.0[ns]	35	186.8 ± 16.5[ns]	−2	105.1 ± 19.4[ns]	2
Lupeol	154.1 ± 13.9[ns]	18	119.9 ± 14.9[ns]	−9	112.4 ± 9.2[ns]	−4
Erythrodiol	104.9 ± 27.5[b]	44	201.0 ± 18.4[ns]	−10	109.9 ± 10.4[ns]	−2
α-Glycyrrhetinic acid	138.4 ± 6.2[ns]	26	137.0 ± 14.5[ns]	25	70.6 ± 21.7[ns]	35
β-Glycyrrhetinic acid	128.9 ± 15.1[ns]	31	107.7 ± 17.0[b]	41	54.0 ± 8.0[a]	50
Hederagenin	157.8 ± 9.5[ns]	16	135.4 ± 20.1[ns]	26	113.8 ± 11.3[ns]	−6
Oleanolic acid	113.8 ± 26.3[a]	40	227.2 ± 6.1[ns]	−24	41.1 ± 11.5[b]	62
α-Amyrin	156.2 ± 10.4[ns]	17	207.9 ± 21.6[ns]	−13	136.0 ± 22.2[ns]	−26
Ursolic acid	164.2 ± 13.9[ns]	13	202.5 ± 15.0[ns]	−10	92.7 ± 9.3[ns]	14
Uvaol	132.8 ± 26.4[ns]	29	164.5 ± 20.0[ns]	10	118.6 ± 19.6[ns]	−10
Dexamethasone	33.1 ± 6.5[b]	82	23.4 ± 6.6[b]	87	9.7 ± 5.0[b]	91

Triterpenoids (0.5 mg per ear) and dexamethasone (0.05 mg per ear) were applied topically 2 h before irritant administration. Ear oedema was measured 4 h after the irritant application. Values are expressed as mean ± S.E.M. for six animals (minimum). Statistical significance of difference from the control [a]$P < 0.05$, [b]$P < 0.01$, [ns]$P > 0.05$. ΔT: Increase in ear thickness. I%: Inhibition percentage.

inhibition was expressed as the percent volume reduction referred to the control group.

2.5. Rat skin inflammation induced by glucose oxidase

The experiment was carried out according to Trenam et al. (1991). The rats were anaesthetised by i.m. administration of 0.25 ml ketamine (100 mg/kg). Oedema was produced on the back, shaved a day earlier, by the intradermal injection of 0.1 ml of a solution containing 1.44 units of glucose oxidase and 64 μg polyethylene glycol 5000 in an ethanol/tween 80/saline (1:1:10) mixture. A blank group was injected with 64 μg polyethylene glycol 5000. Triterpenoids (0.25 mg per site) were dissolved in ethanol/tween 80/saline (1:1:10) and co-injected with glucose oxidase–polyethylene glycol 5000. The standard drug was catalase (0.3 μg per site)–polyethyleneglycol 5000. Oedema was quantified by measuring the doubled thickness of the skin in the inflamed area, using a gauge caliper, 6 h after the glucose oxidase injection. The percent oedema inhibition was expressed as the difference between the control (glucose oxidase group) value and the mean values for each group, divided by the difference between the control and blank group values.

2.6. Statistical analyses

The extent of the oedema was expressed as mean ± S.E.M. Inhibition percentages were calculated from the differences between drug-treated and non-treated tissues, referred to the control treated only with the inflammatory agent. One-way analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons of unpaired data was used for statistical evaluation. Single comparison of groups in the dose–response studies with DPT and DPP was performed by applying Bonferroni's test.

3. Results

The structural formulae of the triterpenoids studied here are shown in Fig. 1a. The compounds belong to three well-defined groups: the lupane-type (betulin, betulinic

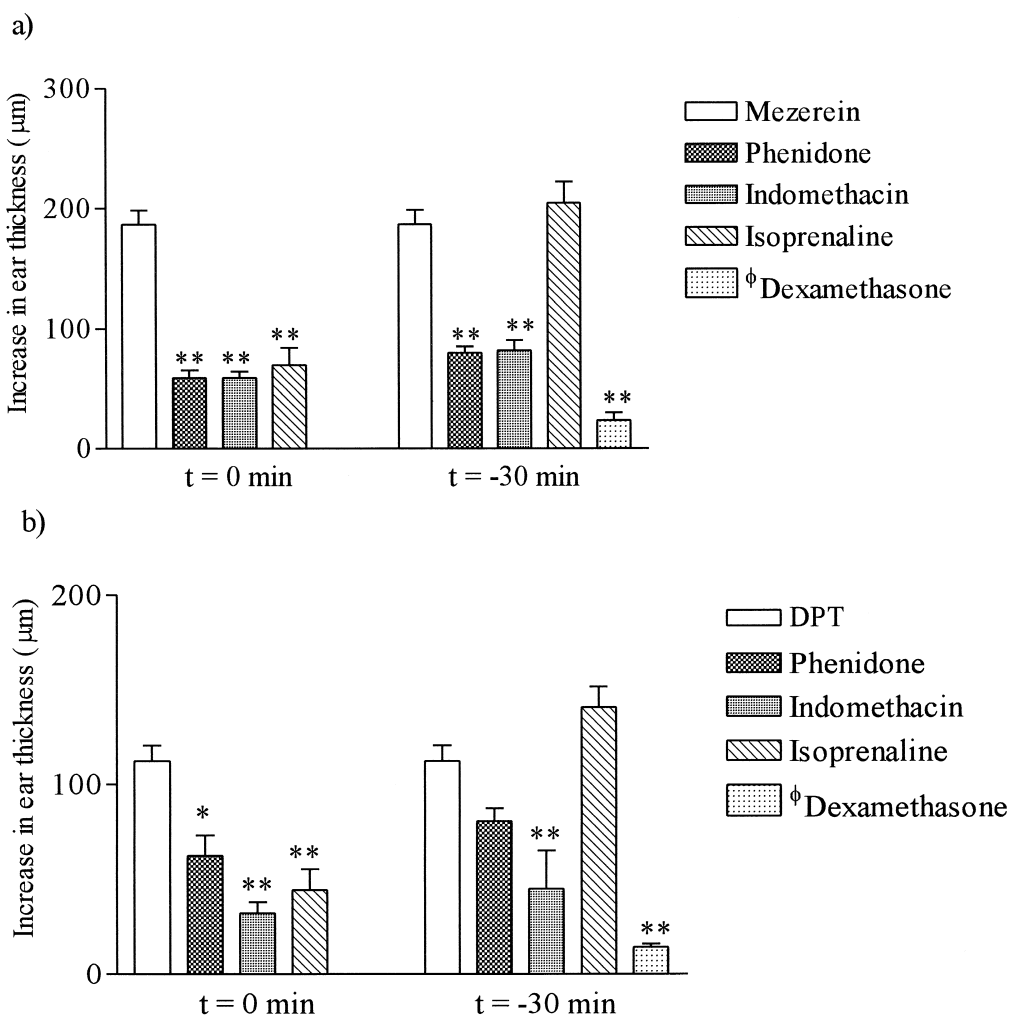


Fig. 2. Anti-inflammatory effect of standard drugs against (a) mezerein and (b) DPT-induced ear oedema administered simultaneously with ($t = 0$ min) or before ($t = -30$ min) irritant agents. Test compounds were assayed at 0.5 mg per ear except dexamethasone that was used at 0.05 mg per ear. ϕCorticoid was applied 2 h before mezerein or DPT. Statistical significance of difference from the control: $^{*}P < 0.05$, $^{**}P < 0.01$.

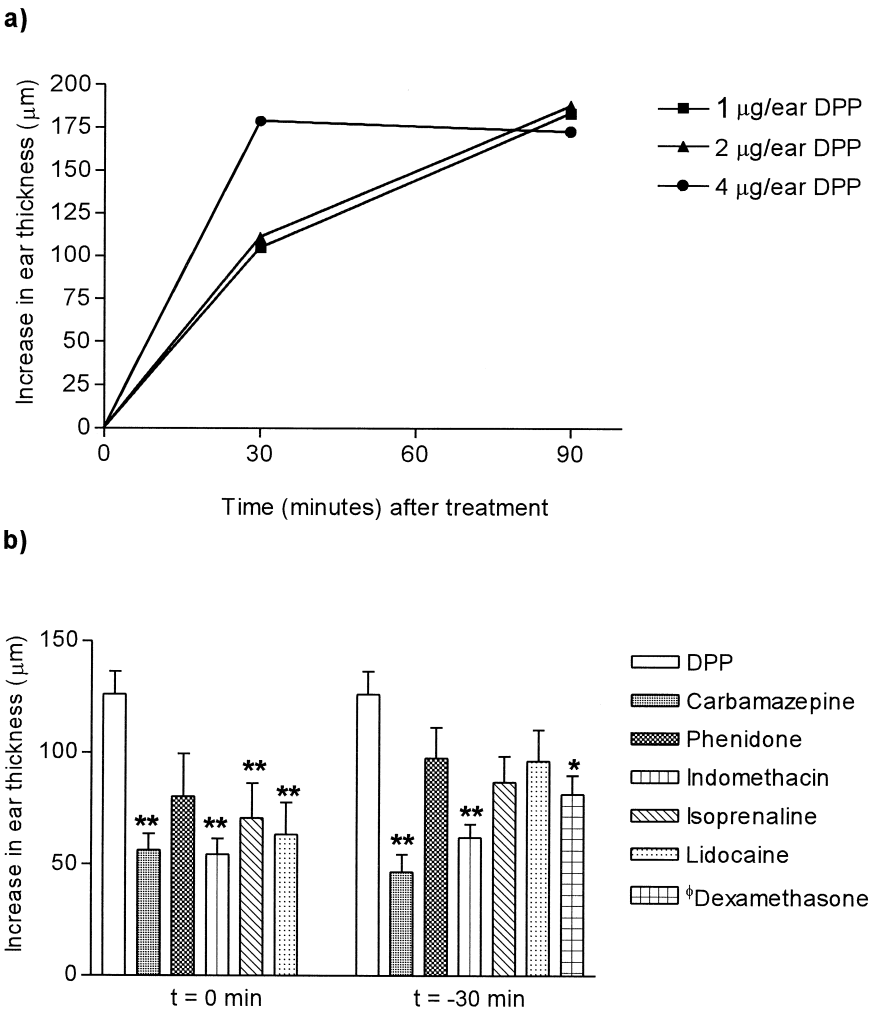


Fig. 3. (a) Time course of ear oedema formation induced by DPP. Each point represents the mean ± S.E.M. value for six animals. (b) Anti-inflammatory effect of standard drugs against DPP-induced ear oedema administered simultaneously with ($t = 0$ min) or before ($t = -30$ min) DPP. Test compounds were assayed at 0.5 mg per ear except dexamethasone that was used at 0.05 mg per ear. ϕCorticoid was applied 2 h before DPP. Statistical significance of difference from the control: $^{*}P < 0.05$, $^{**}P < 0.01$.

acid and lupeol), oleanane-type (erythrodiol, 18α- and 18-β-glycyrrhetinic acids, hederagenin and oleanolic acid), and ursane-type (α-amyrin, ursolic acid and uvaol). Fig. 1b shows the structures of tigliane- and daphnane-based inflammatory diterpenoid esters.

3.1. Mouse ear oedema induced by mezerein

Of the triterpenoids studied, three, namely erythrodiol, lupeol and ursolic acid, showed percentages of oedema inhibition in the same range that of indomethacin (Table 1), although they were fairly inactive when administered 2 h before the inflammatory agent. In this case, only β-glycyrrhetinic acid was able to significantly inhibit the development of the oedema. Hederagenin also showed an improved effect when administered prior to mezerein, though the results were not significant (Table 2). The effects of indomethacin and phenidone, which are classified, respectively, as inhibitors of cyclooxygenase and lipoxygenase activities, on the mezerein-induced oedema were equivalent, not only when the compounds were administered together with mezerein, but also when they

Table 3
ID_{50} values for active triterpenoids and indomethacin on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-, mezerein-, 12-deoxyphorbol-13-tetradecanoate (DPT)- and 12-deoxyphorbol-13-phenylacetate (DPP)-induced ear oedema

	ID_{50} (μmol per ear)			
	TPA	Mezerein	DPT	DPP
Betulin	nd	nd	1.01 (0.80–1.32)	nd
Betulinic acid	nd	nd	nd	0.77 (0.55–1.23)
Erythrodiol	0.35 (0.19–0.54)	1.18 (0.93–1.32)	nd	nd
Indomethacin	0.29[a]	0.80 (0.5–1.02)	0.6 (0.013–0.019)	0.94 (0.52–1.19)

nd = not determined. ID_{50} values are reported together with 95% confidence intervals.
[a] Literature data (Prieto et al., 1996).

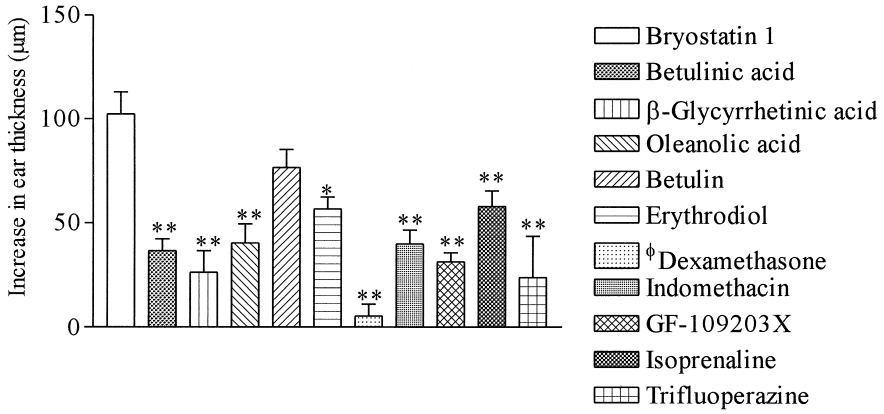


Fig. 4. Anti-inflammatory effect of triterpenoids and standard drugs against bryostatin 1-induced ear oedema. Triterpenoids, indomethacin, isoprenaline and trifluoperazine were assayed at 0.5 mg per ear while dexamethasone and GF-109203X were used at 0.05 mg per ear. Test compounds were administered simultaneously with the inflammatory agent. $^{\phi}$Dexamethasone was applied 2 h before bryostatin 1. Statistical significance of difference from the control: $^{*}P < 0.05$, $^{**}P < 0.01$.

were administered 30 min before mezerein. In the latter case their effects were somewhat lower. However, isoprenaline, which was used because β-adrenoceptor agonists inhibit the oedema induced by many inflammatory agents

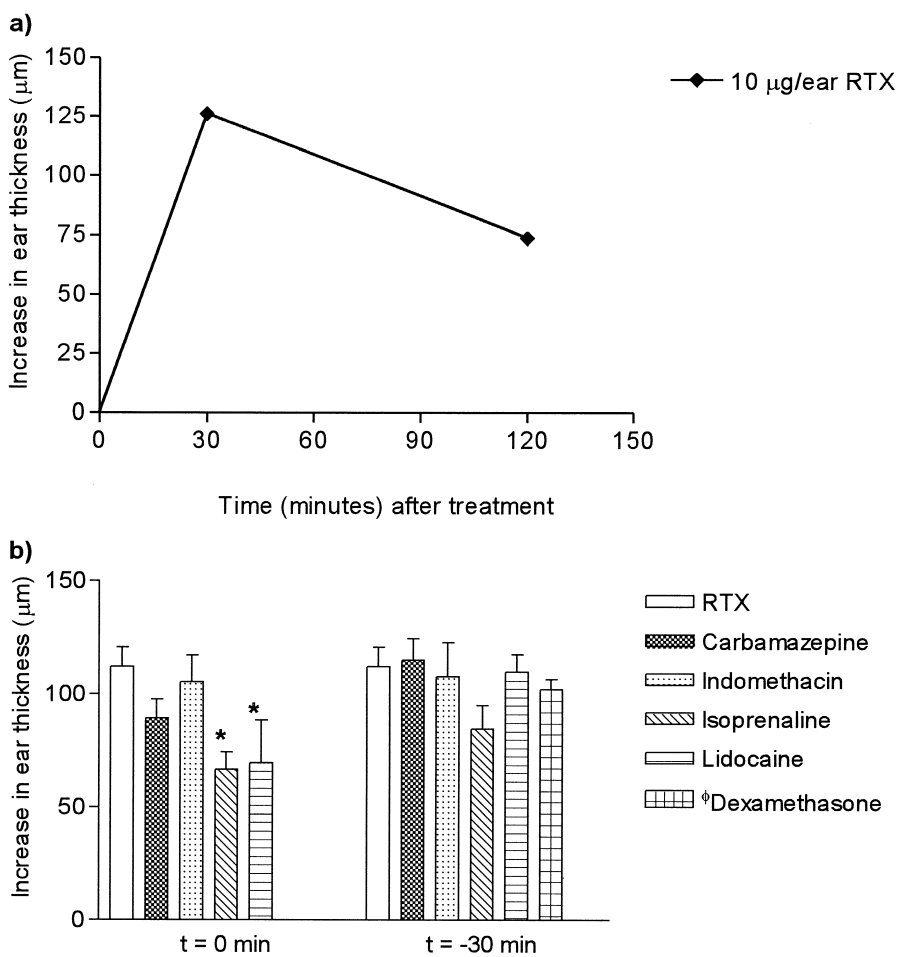


Fig. 5. (a) Time course of ear oedema formation induced by resiniferatoxin (RTX). Each point represents the mean ± S.E.M. value for six animals. (b) Anti-inflammatory effect of standard drugs against resiniferatoxin-induced ear oedema, administered simultaneously with ($t = 0$ min) or before ($t = -30$ min) RTX. Test compounds were assayed at 0.5 mg per ear except dexamethasone that was used at 0.05 mg per ear. $^{\phi}$Corticoid was applied 2 h before neurogenic inflammatory agent. Statistical significance of difference from the control: $^{*}P < 0.05$, $^{**}P < 0.01$.

(Ohuchi et al., 1990), was absolutely ineffective when administered 30 min before mezerein. The 2-h pre-treatment with the glucocorticoid standard, dexamethasone, gave the greatest reduction in the oedema (Fig. 2a).

3.2. Mouse ear oedema induced by DPT and TPA

Prior to studying the effects of the drugs on the oedema induced by DPT, we studied the time courses at different doses. For both +2 h and +4 h determinations, there was a dose-related response, which was always greater after the 4-h interval, although the differences between the effects of the three doses were not significant for either of the times. Seven of the eleven triterpenoids reached inhibitory percentages higher than 40%, especially betulinic acid, betulin and hederagenin (Table 1). Nevertheless, with the 2-h pre-treatment regime applied, oleanolic acid was the most effective, while betulin and β-glycyrrhetinic acid were the only others with any activity (Table 2). When tested against the oedema induced by the 12-esterified analogue, TPA, two triterpenoids, betulin and erythrodiol, caused significant reductions (over 40%) of the oedema (Table 2). When the standard drugs were used against 1 μg DPT, indomethacin inhibited more strongly than phenidone, and isoprenaline lost its effect when administered 30 min before DPT. Again, the 2-h pre-treatment with dexamethasone produced the greatest oedema inhibition (Fig. 2b).

3.3. Mouse ear oedema induced by DPP

The three different doses of DPP gave equivalent increases in ear thickness after 90 min, but a 4 μg dose of DPP produced a much greater response after 30 min (Fig. 3a). The effects of triterpenoids in this model were moderate (roughly in the range of 30–40% inhibition) and not statistically significant, with the exception of those of betulinic and oleanolic acids (Table 1). When a series of standards were applied simultaneously with 4 μg of DPP, the greatest effect was produced by indomethacin and by two neuronal membrane stabilisers, carbamazepine and lidocaine. The efficacy of carbamazepine improved when it was applied 30 min earlier, whereas the others were less effective (Fig. 3b).

The 50% inhibitory dose (ID_{50}) was determined, for indomethacin against mezerein-, DPT- and DPP-induced ear oedema, and for erythrodiol against TPA- and mezerein-, betulin against DPT-, and betulinic acid against DPP-induced ear oedema. The highest potency for indomethacin was observed against DPT, and the triterpenoids had ID_{50} in the range of that of indomethacin in the different models (Table 3).

3.4. Mouse ear oedema induced by bryostatin 1

Five of the triterpenoids that yielded the best results against inflammatory phorbol esters, together with five standard drugs, were tested for their inhibitory activity against the ear oedema in response to bryostatin 1. Be-

Table 4
Effect of triterpenoids as simultaneous or 30-min pretreatment on resiniferatoxin and xylene-induced ear oedema

	$T = 0$				$T = -30$ min			
	Resiniferatoxin		Xylene		Resiniferatoxin		Xylene	
	ΔT (μm)	I%	ΔT (μm)	I%	ΔT (μm)	I%	ΔT (μm)	I%
Control	126.0 ± 19.5		178.2 ± 8.3		159.9 ± 10.3		153.6 ± 25.8	
Betulin	93.1 ± 8.2^{ns}	26	187.3 ± 23.2^{ns}	−5	154.7 ± 19.7^{ns}	3	114.0 ± 17.4^{ns}	26
Betulinic acid	97.4 ± 5.1^{ns}	23	148.9 ± 15.8^{ns}	16	139.3 ± 18.7^{ns}	13	190.3 ± 30.9^{ns}	−24
Lupeol	121.8 ± 10.5^{ns}	3	184.2 ± 16.3^{ns}	−3	160.9 ± 17.8^{ns}	−7	152.2 ± 33.2^{ns}	1
Erythrodiol	109.9 ± 15.6^{ns}	13	176.6 ± 20.2^{ns}	1	161.9 ± 19.8^{ns}	−7	153.8 ± 25.5^{ns}	0
α-Glycyrrhetinic acid	131.5 ± 10.2^{ns}	−4	167.7 ± 7.8^{ns}	6	169.1 ± 8.3^{ns}	−6	169.7 ± 14.3^{ns}	−11
β-Glycyrrhetinic acid	120.3 ± 15.8^{ns}	5	203.4 ± 27.3^{ns}	−14	108.9 ± 10.4^{ns}	32	132.7 ± 20.5^{ns}	14
Hederagenin	86.0 ± 6.2^{ns}	32	161.5 ± 20.0^{ns}	9	176.0 ± 12.5^{ns}	−10	184.4 ± 17.9^{ns}	−20
Oleanolic acid	147.9 ± 13.7^{ns}	−17	190.6 ± 24.4^{ns}	−7	174.1 ± 15.9^{ns}	−9	174.7 ± 15.6^{ns}	−14
α-Amyrin	125.3 ± 14.9^{ns}	1	174.0 ± 5.0^{ns}	2	155.3 ± 17.8^{ns}	3	151.3 ± 17.8^{ns}	2
Ursolic acid	130.1 ± 9.8^{ns}	−3	109.8 ± 11.8^{ns}	38	139.2 ± 18.1^{ns}	13	141.4 ± 18.0^{ns}	8
Uvaol	127.0 ± 12.0^{ns}	−1	166.0 ± 17.9^{ns}	7	138.7 ± 13.8^{ns}	13	91.1 ± 11.5^{ns}	41
Capsazepine	102.1 ± 7.3^{ns}	19	n.t.	–	80.5 ± 8.4^{b}	50	n.t.	–
Isoprenaline	71.3 ± 6.4^{a}	43	105.4 ± 15.5^{ns}	28	93.2 ± 10.5^{a}	42	59.8 ± 26.0^{a}	61
Lidocaine	69.5 ± 18.9^{a}	38	84.7 ± 9.5^{b}	53	n.t.	–	n.t.	–

Test compounds were applied topically (0.5 mg per ear) simultaneously with the irritants ($t = 0$) or 30 min before neurogenic irritants ($t = -30$ min). Ear oedema was measured 30 min after resiniferatoxin (0.01 mg per ear) or xylene (20 μl per ear) application. Values are expressed as mean ± S.E.M. Statistical significance of difference from the control: $^{a}P < 0.05$, $^{b}P < 0.01$, $^{ns}P > 0.05$. ΔT: Increase in ear thickness. I%: Inhibition percentage. n.t.: not tested.

tulinic and oleanolic acids showed an effect similar to that of indomethacin, whereas the triterpene alcohols, erythrodiol and betulin, were less active, all at the same dose, 0.5 mg per ear (Fig. 4). With the exception of the greater effect of dexamethasone, some known protein kinase C inhibitors with different selectivities such as trifluoperazine, GF-109203X and β-glycyrrhetinic acid were more effective than indomethacin and isoprenaline.

3.5. Mouse ear oedema induced by resiniferatoxin and xylene

The ear oedema induced by resiniferatoxin, which was characterised by an early peak that declined after 2 h (Fig. 5a), was reduced significantly only by previous administration of the competitive antagonist, capsazepine (Table 4), or by simultaneous administration of isoprenaline and lidocaine (Fig. 5b). The oedema induced by xylene plateaued within the first hour after the application (Fig. 6a). In this case, isoprenaline and carbamazepine were active when applied 30 min before the irritant (Fig. 6b). None of the triterpenoids was able to modulate significantly the oedema induced by either resiniferatoxin or xylene (Table 4).

3.6. Mouse ear oedema induced by arachidonic acid

The only triterpenoid active against arachidonic acid-induced oedema, was erythrodiol, which produced 38% inhibition ($P < 0.05$). The other triterpenes, namely betulin, betulinic acid, lupeol, α- and β-glycyrrhetinic acids, hederagenin, oleanolic acid, α-amyrin, ursolic acid and uvaol failed to inhibit the oedema significantly. Indomethacin and phenidone gave 60% and 52% inhibition, respectively ($P < 0.01$), and nordihydroguaiaretic acid inhibited by 45% ($P < 0.05$).

3.7. Bradykinin-induced mouse paw oedema

Several triterpenoids were effective on the paw swelling induced by bradykinin. Erythrodiol, applied 30 min before the inflammatory, showed greater activity than isoprenaline, and four other compounds inhibited the oedema

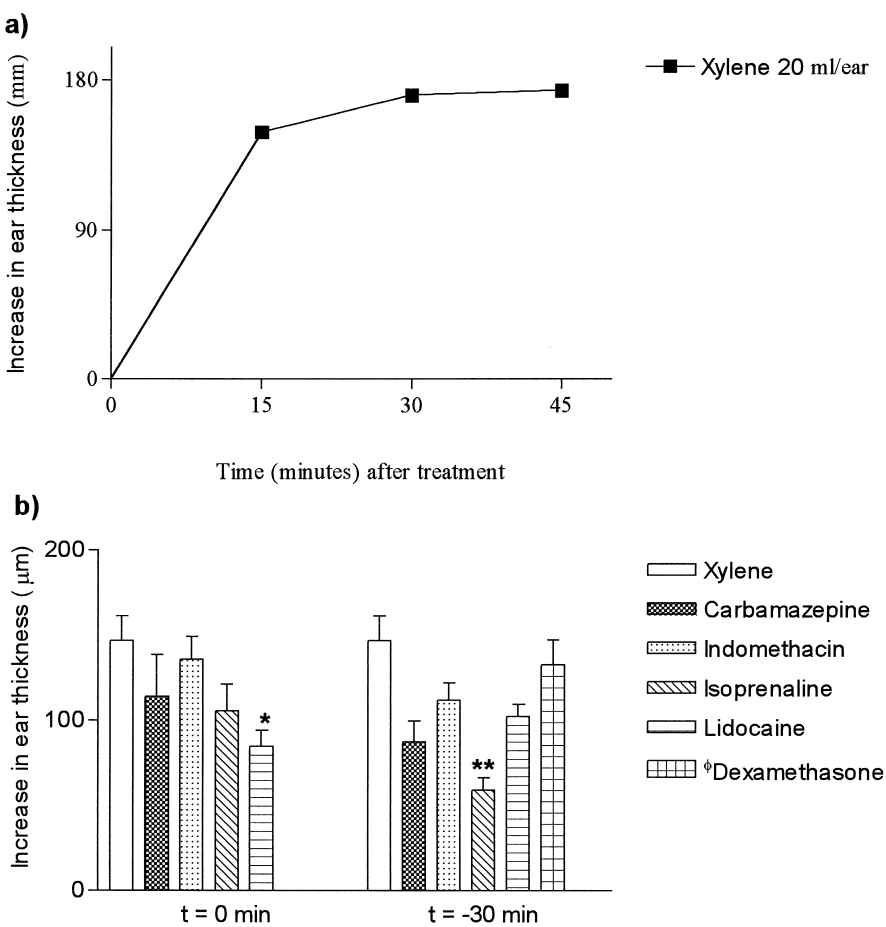


Fig. 6. (a) Time course of ear oedema formation induced by xylene. Each point represents the mean ± S.E.M. value for six animals. (b) Anti-inflammatory effect of standard drugs against xylene-induced ear oedema, administered simultaneously with ($t = 0$ min) or before ($t = -30$ min) xylene. Test compounds were assayed at 0.5 mg per ear except dexamethasone that was used at 0.05 mg per ear. ΦCorticoid was applied 2 h before neurogenic inflammatory agent. Statistical significance of difference from the control: $^{*}P < 0.05$, $^{**}P < 0.01$.

Table 5
Effect of triterpenoids as 30-min or 1-h pretreatment on bradykinin-induced mouse paw oedema

	$T=-30$ min		$T=-1$ h	
	ΔV (μl)	I%	ΔV (μl)	I%
Control	67 ± 2		65 ± 9	
Betulin	37 ± 6^{b}	45	30 ± 8^{a}	54
Betulinic acid	63 ± 2^{ns}	6	30 ± 6^{a}	54
Lupeol	65 ± 4^{ns}	2	42 ± 10^{ns}	35
Erythrodiol	18 ± 8^{b}	73	17 ± 0^{b}	74
α-Glycyrrhetinic acid	38 ± 8^{b}	43	30 ± 8^{a}	54
β-Glycyrrhetinic acid	50 ± 4^{ns}	25	58 ± 7^{ns}	11
Hederagenin	58 ± 3^{ns}	13	48 ± 7^{ns}	26
Oleanolic acid	30 ± 4^{b}	55	36 ± 10^{ns}	45
α-Amyrin	37 ± 6^{b}	45	30 ± 7^{a}	54
Ursolic acid	57 ± 5^{ns}	15	28 ± 8^{a}	57
Uvaol	50 ± 3^{ns}	25	60 ± 6^{ns}	8
Bradykinin antagonist	33 ± 8^{b}	51	–	–
Isoprenaline	28 ± 3^{b}	58	33 ± 6^{a}	49

Triterpenoids (10 mg/kg) and isoprenaline (5 mg/kg) were administered intraperitoneally 30 min or 1 h before irritant treatment. Bradykinin antagonist (0.01 mg per site) was administered together with bradykinin. Paw oedema was measured 12 min after irritant injection. Values are expressed as mean ± S.E.M. for six animals. Statistical significance of difference from the control: $^{a}P<0.05$, $^{b}P<0.01$, $^{ns}P>0.05$. ΔV: Increase in paw volume. I%: Inhibition percentage.

significantly (40–55%). Slightly greater though generally less significant effects were observed with a 1-h interval between drug administration and bradykinin injection (Table 5). With a 3-h interval erythrodiol and ursolic acid still showed some activity, although the inhibitory effect was not significant. Dexamethasone reduced the swelling by 59% ($P<0.05$).

3.8. Rat skin inflammation induced by glucose oxidase

When the various triterpenoids were tested on the model of intradermal inflammation caused by nascent hydrogen peroxide, the alcohols, betulin and erythrodiol, were the most active, while the triterpene acids seemed to have similar efficacy. The exception was ursolic acid, which reached only one third of the possible inhibition (Fig. 7).

4. Discussion

All the protein kinase C activators used in the present work as inflammatories, except bryostatin 1, have a diterpenoid skeleton and fit the definition of *phorbol esters* and congeners given in the Introduction. Three of these activators, TPA, DPT and DPP, are truly phorbol derivatives, whereas mezerein is a daphnane ester containing a typical trioxygenated quaternary carbon linked to a phenyl group.

Because of the observed effect of topically administered indomethacin and phenidone on the swelling induced by mezerein, it can be deduced that both cyclooxygenase- and lipoxygenase-mediated arachidonic acid metabolism pathways must be similarly involved in the inflammatory process. Co-administration of isoprenaline, which acts by increasing the levels of cAMP in microvascular endothelial layers (Vigne et al., 1994), is as effective as that of indomethacin, whereas pre-treatment had no effect.

If we compare the anti-oedema effect of the triterpenoids administered simultaneously against mezerein, it can be deduced that neither a certain basic skeleton nor carboxyl groups exert any influence on the activity. After pre-treatment 2 h earlier, a delay usually applied for glucocorticoids, β-glycyrrhetinic acid showed the greatest oedema inhibition. This acid has a 11-keto group and is widely reported to be a strong inhibitor of the activity of 11-β-hydroxysteroid-dehydrogenase and 11-β-hydroxysteroid-dehydrogenase-mRNA synthesis (Whorwood et al., 1993).

Concerning the time course studies of the DPT-induced oedema, in spite of DPT being a 12-deoxy derivative, a feature shared by the phorbol esters producing oedema with the earliest maximum, we found the swelling to be up to 10 times greater after 4 h than after 2 h, possibly because of the length of the ester side chain (Hergenhahn et al., 1982). This is similar to what happens with TPA, which is the prototypical 2,13-dioxy derivative. As the slope of the dose–response relationship was low, the intermediate dose (1 μg per ear) was chosen. As to the effect of the standard drugs tested against DPT-induced oedema,

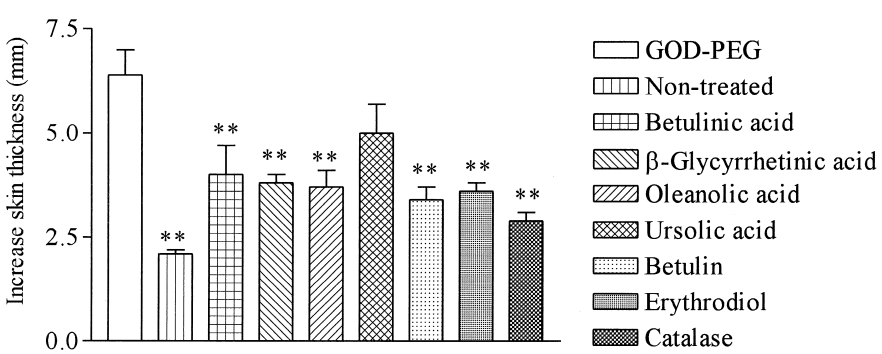


Fig. 7. Anti-inflammatory effect of selected triterpenoids (0.250 mg per site) and catalase (0.3 μg per site) against the intradermal inflammation induced by glucose oxidase (GOD). Statistical significance of difference from the control: $^{*}P<0.05$, $^{**}P<0.01$.

the main difference with respect to mezerein was that lipoxygenase-mediated arachidonic acid metabolism has a minor influence, because phenidone appeared to be comparatively less effective.

Two of the compounds found to be inactive against mezerein, oleanolic acid and α-amyrin, had moderate efficacy against DPT-induced oedema. Some of the other triterpenoids, especially lupanes (betulin, betulinic acid and lupeol), showed comparable increases in effect, although others, such as ursolic acid, were less active. With a 2-h pre-treatment, all the triterpenoids lost most of their effect, except α-glycyrrhetinic, β-glycyrrhetinic and oleanolic acids. Together, these results indicate that, in comparison with lupanes and ursanes, an oleanane skeleton is favourable for activity against DPT.

We described the effect of simultaneous administration of the test compounds against TPA-induced oedema earlier (Recio et al., 1995b). Now we showed that a general diminution of the efficacy occurs when the pre-treatment schedule was applied, although the average effect of the triterpenoids on TPA-induced oedema was consistently much greater than that against mezerein and DPT under the same conditions.

The application of a set of standard drugs to the model of ear oedema induced by bryostatin 1 allowed us to survey the possibilities of counteracting the inflammatory effects of this macrolactone. We have demonstrated that glucocorticoids and inhibitors of protein kinase C are the most active in this test. Trifluoperazine, which is known to act as a protein kinase C inhibitor mainly by interacting with the phospholipid cofactor (Wise and Kuo, 1983), does not seem to be as potent as the selective inhibitor, the bisindolylmaleimide derivative GF-109203X, for although both compounds reached similar percentages of oedema reduction, GF-109203X was administered at one-tenth of the dose used for trifluoperazine. The effect of indomethacin was of the same magnitude as that against DPT and mezerein, whereas that of isoprenaline clearly was not. This suggests a long-lasting (16 h) involvement of cyclooxygenase products in the bryostatin 1-induced oedema. From the results obtained with triterpenoids on the effect of bryostatin 1, we deduce that oleanane derivatives are more active than lupane derivatives and that the replacement of hydroxymethyl groups by carboxyl groups (erythrodiol/oleanolic acid and betulin/betulinic acid) increases the effect. It is consistent with previous reports on the inhibitory effect of glycyrrhetinic acid on protein kinase C activity (O'Brian et al., 1990) and TPA-induced oedema (Recio et al., 1995b) that this compound showed the highest effect among the triterpenoids tested.

DPP is an irritant, non-promoter phorbol derivative without the long alkyl chain substitution of TPA or DPT that induces a much shorter lasting oedema than that induced by other classic related compounds. In our experiments, the partial neurogenic character of the inflammatory response to this agent, which was hypothesised by Szallasi and Blumberg (1989), seems to be confirmed by the marked effect of neuronal Na^+ channel blockers such as carbamazepine and lidocaine, although the latter compound reduces the extravasation, thus also acting on endothelial cells (Dux et al., 1996). The duality of the mechanisms driven by DPP is also consistent with the fact that the potency of DPP must be different for its immediate (neurogenic) effects and for its delayed (non-neurogenic, protein kinase C-related) effects, as is suggested by the lack of dose–response relationship at 90 min (see Fig. 3a). As the effect of triterpenoids was mild, they presumably do not interfere with neuronally mediated inflammation. When the preponderance of neurogenic character prevails, as in the effects of resiniferatoxin and xylene, the activity of triterpenoids diminishes drastically, which must imply a lack of effect on capsaicin receptors or on neuropeptide release, particularly substance P, neurokinins or calcitonin gene-related peptide release (Barnes et al., 1990). Capsazepine is a competitive antagonist of the vanilloid receptor, as demonstrated in several in vitro studies (Bevan et al., 1992). We now describe its topical anti-inflammatory activity against resiniferatoxin. It has been observed that capsazepine needs a minimum delay after its administration to produce an effect, which might indicate that it binds to, or reaches, the receptor more slowly than does resiniferatoxin. The lack of effect of dexamethasone in the case of resiniferatoxin is similar to what was previously seen for betamethasone-17-valerate against the skin blood flow increase induced by capsaicin (Ahluwalia and Flower, 1993).

The effect of isoprenaline on the oedema induced by resiniferatoxin and xylene was the greater among those observed for 30-min pre-treatment with the various drugs. The low level effects of most classical anti-inflammatory drugs under the same conditions suggest that modulation of neurogenic oedema may occur through an ultimate event such as retraction of capillary endothelial cells allowing leakage of fluids. This hypothesis can be supported by the fact that the β_2-adrenoceptor agonist, formoterol, inhibits the paracellular gap formation induced by substance P and electrical vagal stimulation (Baluk and McDonald, 1994), and isoprenaline itself increased the number of endothelial tight junctions with respect to the basal levels (Adamson et al., 1998).

As the inflammation caused by bradykinin in the mouse foot pad depends on three different events: release of arachidonic acid and of neuropeptides (Campos and Calixto, 1995), and activation of protein kinase C (Tippmer et al., 1994), it is not easy to correlate the marked effect of some triterpenoids against bradykinin with these mechanisms. However, given the results seen for erythrodiol, betulinic and oleanolic acids on the inflammation induced by phorbols and bryostatin 1, a direct relationship between inhibition of protein kinase C-related effects and inhibition of bradykinin oedema is proposed. Alcoholic triterpenoids were in general more active than acidic ones, whereas

acids such as betulinic and ursolic have an increased effect when pre-treatment is at 1-h interval. In the case of ursolic acid, this may correlate, with its recently described property of inhibiting cyclooxygenase-2 activity in a time-dependent manner (Ringbom et al., 1998). The effect of the bradykinin B_1 receptor antagonist, [Leu^8]des-Arg^9-bradykinin, injected simultaneously with bradykinin indicates that this receptor is not exclusively inducible, as was analogously found in the mouse pleural cavity (Pesquero et al., 1996; Hess et al., 1996).

The anti-oedematous effect of the triterpenoids selected to modulate in situ the hydrogen peroxide-induced inflammation in rat skin did not appear to be dependent on chemical structure. As a relatively strong activity was observed for the compounds that also showed activity in the mouse ear models, it seems that blocking reactive oxygen species generation is a possible mechanism for ear oedema reduction. Further analysis to ascertain whether triterpenoids inhibit the activity of glucose oxidase or act as reactive oxygen species scavengers is required.

In conclusion, it has been proven that many natural triterpenoids are efficient anti-inflammatory drugs when applied topically against various protein kinase C activators, such as phorbol esters and bryostatin 1. Those triterpenoids with oleanane-based or lupane-based structures are, in general, more active than ursane-based ones. As most of them are inactive against the inflammation induced by arachidonic acid and in neurogenic inflammatory models, their effects may depend on in vivo inhibition of protein kinase C, an effect reported for some members of this class of compounds in vitro (Wang and Polya, 1996). On the other hand, the inhibition of free radical generation should be quantified to establish its possible contribution to the anti-oedema activity.

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