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The potent irritancy of the daphnane orthoester, resiniferatoxin, exhibits features of a mixed aetiology

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Abstract—Resiniferatoxin-induced erythema of mouse ear was shown to possess characteristics of both a phorbol ester-mediated response and that induced by the neurogenic irritant, capsaicin. Whereas the response to the phorbol ester, sapintoxin D, was delayed and prolonged, and was augmented by capsaicin pretreatment, the response to resiniferatoxin was biphasic, with the early phase being antagonized by capsaicin desensitization. However, resiniferatoxin was most potent in inducing a delayed erythema which, unlike the capsaicin response, was sensitive to inhibition by low dose hydrocortisone treatment, but not to chronic capsaicin desensitization. It is concluded that the erythema response to resiniferatoxin has a mixed aetiology, which may explain the unique potency of this toxin.

Diterpene ester toxins, notably the phorbol ester and tigliane groups, are most widely known as tumour promotors and potent activators of protein kinase C (PKC); however, inflammation is the most general and acutely potent property of these compounds (Evans & Edwards 1987). In the daphnane orthoester series, resiniferatoxin (9,13,14-orthophenylacetylresiniferonol-20-*O*-homovanillate) (Rx) (Fig. 1a) is of particular interest. This compound is the most potent diterpene ester irritant so far characterized, yet it barely activated PKC from rat brain in-vitro and is not a tumour promotor (Hausen et al 1979). Rx differs from PKC-activating compounds in lacking a free C-20 hydroxyl group. Since the homovanillate substitution at this position resembles the polar head group of the neurotoxin, capsaicin, it has been suggested that Rx can act as an ultrapotent analogue, inducing neurogenic inflammation and hypothermia (de Vries & Blumberg 1989; Szallasi & Blumberg 1989). At the same time, we have identified a Ca^{2+}-independent kinase activity in mononuclear cells which is stimulated to a greater extent by Rx than by phorbol esters, is not significantly activated by capsaicin, and which can be separated from known isozymes of PKC by hydroxyapatite chromatography (Ryves et al 1989).

The established technique for quantitative estimation of diterpene ester irritant potency, the mouse ear erythema test, has previously been used to classify compounds into distinct irritant groups on the basis of duration and latency of response (Evans & Schmidt 1979). In this study the polar 12-tetradecanoylphorbol-13-acetate/analogue, sapintoxin D (Fig. 1b), showed a potent response of rapid onset and short duration, similar to that observed for resiniferatoxin. Since this phorbol ester is equipotent with TPA in activating PKC in-vitro it was decided to compare the compounds with capsaicin in more detail using the erythema assay.

Materials and methods

Test agonists and antagonists. Rx and sapintoxin D were isolated from plant sources *Euphorbia poisonii* and *Sapium indicum*, respectively, by centrifuged liquid chromatography, and their structures verified by NMR and mass spectrometry (Evans 1986). Capsaicin, hydrocortisone and indomethacin were obtained from Sigma, (Poole, UK). Compounds were diluted in acetone (sapintoxin D, capsaicin, Rx) or ethanol (hydrocortisone, indomethacin), immediately before testing.

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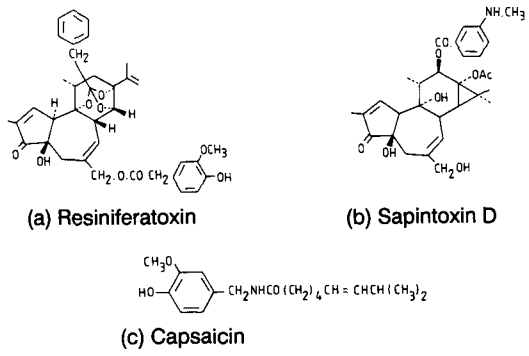

FIG. 1. Structures of diterpene ester irritants.

Mouse ear erythema test. Female BKTO mice (B & K Ltd, Hull, UK) (~20 g) were assigned randomly to groups of 10 animals each, and 5 μL of the test agonist applied to the inner surface of the right ear of each mouse. Five μL of control diluent was applied to the left ear. Colour changes in the ears were assessed by an independent observer at regular intervals and any comparative reddening of the right ear scored positive. From a twofold dilution test series, the minimum dose consistently (>99%) producing response in all animals was determined, and used for inhibitor studies. Inhibitors were applied in 5 μL ethanol to both ears 30 min before agonist application.

Results and discussion

In agreement with previously published results (Evans & Schmidt 1979), Rx was an extremely potent inducer of erythema in the mouse ear test, being approximately 20-fold more potent than sapintoxin D and almost 100-fold more potent than capsaicin on a weight basis (Table 1). The duration of the Rx response more closely resembled that of sapintoxin D, in that at maximally effective doses, it was prolonged (> 3 h) and involved a generalized ear reddening. At higher doses, the response nevertheless had a shorter duration than that due to sapintoxin D. In contrast, the response to capsaicin could not be prolonged much beyond 1 h, and at the minimum 100% effective dose,

Table 1. Comparison of potency and duration of response.

	Minimum dose producing maximal (100%) response		Maximum dose tested	
Compound	(μg mL^{-1})	Duration	(μg mL^{-1})	Duration
Capsaicin	12·5	11 (±2) min	7000	80 (±13) min
Resiniferatoxin	0·16	>3 h	1000	>8 h
Sapintoxin D	2·8	>3 h	75	>12 h

Data from four experiments.

consisted of a transitory vasodilation without generalized erythema.

Since latency is a typical feature of phorbol ester-induced irritancy, the times taken to reach peak irritancy at selected, maximally effective doses were compared (Fig. 2). Even at the minimal 100% effective dose, the response to capsaicin was almost immediate, whereas the sapintoxin D response took over 30 min to develop at all doses tested. In the case of Rx however, the degree of latency was distinctly dose-dependent. At the lowest 100% effective dose, the response peaked at 48 ±6 min, a slightly more rapid onset than the sapintoxin D response, but significantly more delayed than the equivalent dose of capsaicin. At higher selected doses, however, an immediate response was obtained.

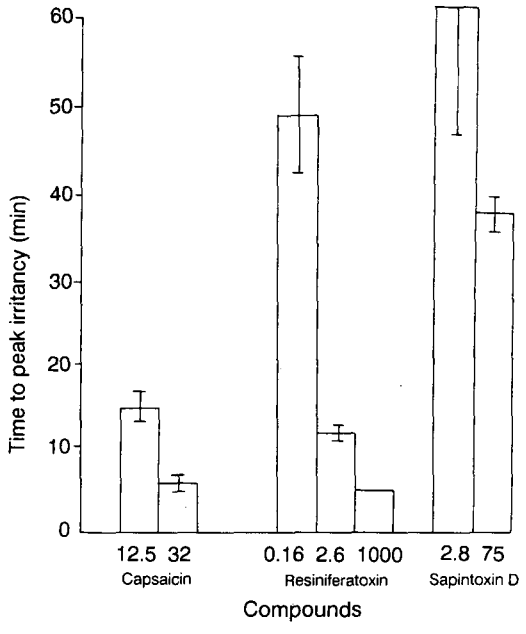

FIG. 2. Effect of dose on time to peak irritancy. Data from 3–5 experiments. Each of lower selected doses ($\mu g\ mL^{-1}$) is the minimum dose producing maximal (100%) response. Higher doses for Rx are those producing duration of response >3 and >8 h, and for sapintoxin D, >12 h, respectively. Error bars show standard error between experiments in which all doses were tested in parallel in groups of 10 animals each.

As previously observed (Bevan et al 1987), repeated capsaicin treatment produced a progressively attenuated response, and ultimately non-responsiveness of the ear (Fig. 3). However, capsaicin pretreatment was capable of dramatically augmenting the response to subsequent application of a subthreshold dose of phorbol ester (Fig. 4).

In the case of Rx, capsaicin pretreatment did not significantly affect potency. However, an interesting observation was the abolition of the dose-dependence of latency (Table 2), with peak irritancy occurring at about 30 min for both high and low dose Rx treatment. The Rx response differed from the capsaicin response in being sensitive to low dose hydrocortisone pretreatment, but was also more sensitive to indomethacin than the phorbol ester. At higher doses of Rx, however, the early response was inhibited by indomethacin treatment only.

The mouse ear erythema assay is a sensitive indicator of irritancy, with an established role in the detection and isolation of diterpene ester toxins from plant extracts. These can be distinguished from natural neurogenic irritants (arylamides, isothiocyanates) on the basis of high potency, sensitivity to inhibitors and latency. This last property does not appear to correlate with any simple parameter of lipophilicity (except for

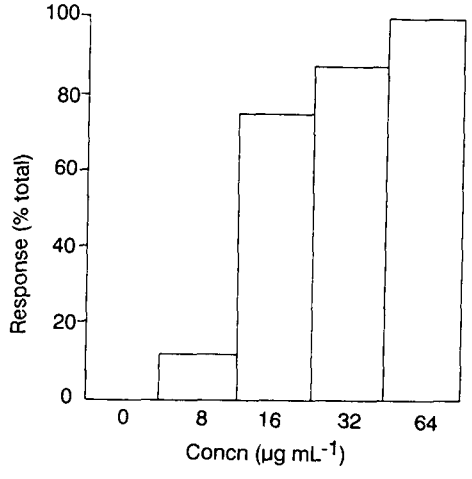

FIG. 4. Synergy of sapintoxin D-induced erythema by capsaicin pretreatment. Data from a single representative experiment. Graph shows % of mice responding (out of 10) to a subthreshold dose of 0·4 mg mL^{-1} sapintoxin D applied 40 min after capsaicin pretreatment.

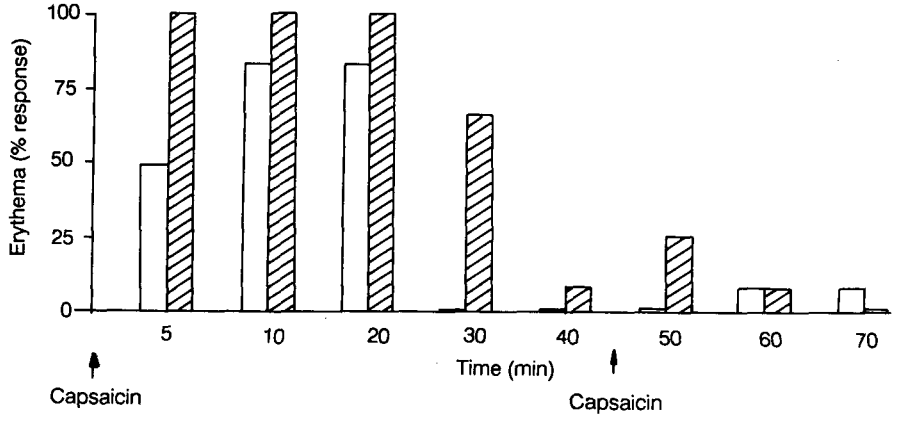

FIG. 3. Erythema response to repeated doses of capsaicin. □ 10, □ 32 mg mL^{-1}.

Table 2. Effect of capsaicin pretreatment on erythema response to resiniferatoxin.

Resiniferatoxin ($\mu g\ mL^{-1}$)	Time to peak irritancy (min ± s.e.)	
	No pretreatment	Capsaicin*
0·16	50 (±7)	28 (+11)
2·6	12 (±1)	28 (±3)

* Fifty $\mu g\ mL^{-1}$ capsaicin applied 50 min before resiniferatoxin treatment. Data from five experiments.

close structural analogues) or permeability barrier, since we have previously shown phorbol ester potency to be unaffected by epidermal stripping (Brooks et al 1988).

The uniquely potent irritant, Rx, despite its structural similarity to the phorbols, has been clearly demonstrated as capable of acting as a capsaicin analogue in several systems (de Vries & Blumberg 1989; Szallasi & Blumberg 1989). In addition, treatment with Rx at doses higher than the maximal response dose induced a rapid onset erythema which was sensitive to indomethacin but not hydrocortisone pretreatment, and to capsaicin pretreatment. Unlike capsaicin, however, at equivalent dose levels, this response progressed into a prolonged, generalized erythema. The late response could be produced independently of any visible rapid onset erythema, and could therefore be regarded as the major irritant action of the compound. As with the phorbol ester response, the late Rx response was sensitive to inhibition with low dose hydrocortisone and its onset was enhanced by capsaicin pretreatment. However, this response was more sensitive to indomethacin than a typical phorbol ester response, and its duration was shorter than that typically induced by the majority of diterpene esters tested to date.

With the exception of 20-substituted derivatives, diterpene ester-induced irritancy correlates well with the ability of these compounds to activate PKC in-vitro (Ellis et al 1987). Rx is capable of low potency, Ca^{2+}-dependent activation of some PKC isozymes, and is the most potent activator of the Ca^{2+}-independent kinase which we term Rx-kinase (Ryves et al 1989). The relative roles of kinase subtypes in mediating diterpene ester-type irritancy remain to be resolved, but the present demonstration of similar features in the response to Rx is consistent with known mechanisms. We would suggest, however, that the extreme potency of this compound is not at present explained on the basis of a single target, and that the characteristics of dose-dependent latency, dual inhibitor sensitivity and interaction with neurogenic irritants point to the existence of a mixed aetiology.

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