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The stimulation of capsaicin-sensitive neurones in a vanilloid receptor-mediated fashion by pungent terpenoids possessing an unsaturated 1,4-dialdehyde moiety

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1 The irritant fungal terpenoid isovelleral caused protective eye-wiping movements in the rat upon intraocular instillation and showed cross-tachyphylaxis with capsaicin, the pungent principle in hot pepper.

2 Isovelleral induced a dose-dependent calcium uptake by rat dorsal root ganglion neurones cultured *in vitro* with an EC_{50} of 95 nM, which was fully inhibited by the competitive vanilloid receptor antagonist capsazepine.

3 Isovelleral inhibited specific binding of [^{3}H]-resiniferatoxin (RTX), an ultrapotent capsaicin analogue, to rat trigeminal ganglion or spinal cord preparations with an IC_{50} of 5.2 μM; in experiments in which the concentration of [^{3}H]-RTX was varied, isovelleral changed both the apparent affinity (from 16 pM to 37 pM) and the co-operativity index (from 2.1 to 1.5), but not the B_{max}.

4 The affinity of isovelleral for inducing calcium uptake or inhibiting RTX binding was in very good agreement with the threshold dose (2.2. nmol) at which it provoked pungency on the human tongue.

5 For a series of 14 terpenoids with an unsaturated 1,4-dialdehyde, a good correlation was found between pungency on the human tongue and affinity for vanilloid receptors on the rat spinal cord.

6 The results suggest that isovelleral-like compounds produce their irritant effect by interacting with vanilloid receptors on capsaicin-sensitive sensory neurones. Since these pungent diterpenes are structurally distinct from the known classes of vanilloids, these data provide new insights into structure-activity relations and may afford new opportunities for the development of drugs targeting capsaicin-sensitive pathways.

Keywords: Isovelleral; pungent terpenoids; unsaturated dialdehydes; resiniferatoxin; capsaicin; capsazepine; vanilloid receptors; novel vanilloids

Introduction

Pungent terpenoids containing an unsaturated 1,4-dialdehyde moiety have been isolated from various natural sources such as higher plants (Kubo *et al.*, 1976; Fukuyama *et al.*, 1982), fungi (Camazine & Lupo, 1984; Sterner *et al.*, 1985), or molluscs (Cimino *et al.*, 1983). Their repellency implies a role in the natural defence systems of their host organisms. Indeed, mammals that normally feed on mushrooms will avoid edible specimens painted with pungent terpenoids (Camazine *et al.*, 1983). As expected, these terpenoids act on a wide range of biological targets, ranging from antimicrobial activity (Anke & Sterner, 1991; Forsby *et al.*, 1991), through an antifeedant action toward the army worm *Spodoptera exempta* (Ma, 1977; Kubo & Nakanishi, 1979), to pungency in mammals, including man (cf Sterner & Anke, 1995). It is very likely that, in order to cause such diverse biological effects, these terpenoids (e.g. isovelleral, polygodial, and warburganal) act on a variety of targets. For example, aphids were shown to detect polygodial with sensilla located on their antennal tips (Powell *et al.*, 1995), whereas warburganal interferes with the stimulus transduction process in the chemoreceptor cells of *Spodoptera exempta* (Ma, 1977; Kubo & Nakanishi, 1979). To identify its primary target(s) in mammals, isovelleral has already been tested against a number of receptors in the central nervous system (CNS): although isovelleral turned out to be a potent inhibitor of dopamine D_1 receptors (Bocchio *et al.*, 1992), this interaction can hardly explain its pungency.

Capsaicin, the primary chemical irritant in hot peppers, likewise causes a burning sensation in the human tongue (Lee, 1954; Jancsó, 1968; Maga, 1975; Rozin & Schiller, 1980), which differs from the effect of other irritants in that it dissipates upon repeated application (Szolcsányi, 1977; Karrer & Bartoshuk, 1991). Capsaicin has long been thought to interact at a specific receptor (Jancsó, 1968; Szolcsányi & Jancsó-Gábor, 1975) the existence of which has recently been demonstrated by the specific binding of [^{3}H]-resiniferatoxin (RTX) (Szallasi & Blumberg, 1990a), a naturally occurring ultrapotent capsaicin analogue (Szallasi & Blumberg, 1989), and by the development of a synthetic, competitive capsaicin antagonist, capsazepine (Bevan *et al.*, 1992; Walpole *et al.*, 1994). As capsaicin and RTX differ dramatically in the rest of the molecule, but share a (homo)vanillyl substituent essential for biological activity, the receptor at which these irritant compounds interact appears to be best described as the vanilloid receptor (cf Szallasi & Blumberg, 1990b; Szallasi, 1994).

Vanilloid receptors represent an attractive target to investigate for chemical irritants of unknown mechanism of action. The majority of terpenoids containing an unsaturated 1,4-dialdehyde functionality are intensely pungent and attack thiol groups or primary amino groups of proteins (Kubo & Ganjian, 1981; Cimino *et al.*, 1987). A correlation between the pungency of both natural and synthetic dialdehydes on the human tongue and their reactivity toward methylamine has been shown (Caprioli *et al.*, 1987). Vanilloid receptors contain thiol groups essential for RTX binding (Szallasi & Blumberg, 1993a), and sulphydryl-reactive agents, such as N-ethylmaleimide, were shown to activate capsaicin-sensitive neurones in cross-tachyphylaxis with capsaicin (Evangelista *et al.*, 1992).

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The aim of the present study was, therefore, to evaluate the possibility that terpenoids containing an unsaturated dialdehyde are pungent because they activate capsaicin-sensitive neurones by interacting at vanilloid receptors.

Methods

Protective eye-wiping movements in the rat upon intraocular instillation

Acute pain-producing potency of isovelleral was determined in the eye-wiping assay of Jancsó and coworkers (1961). Briefly, increasing concentrations (starting at 40 nM) of isovelleral in 10% EtOH/10% Tween80/80% physiological saline were instilled into the eyes of Sprague-Dawley rats (8 females, weighing 200–250 g) and the number of protective wiping movements with the forelegs was counted. To avoid unnecessary discomfort to the animals, the experiment was concluded when the concentration resulting in a reproducible response of at least 10 wiping movements was achieved. Another group of 8 rats received intraocular treatment with a 33 μM capsaicin solution (solvent as for isovelleral), which produced a similar response in previous experiments (Szallasi & Blumberg, 1989). After treatment, both isovelleral- and capsaicin-treated animals were divided into two groups and challenged 6 h later with either isovelleral (400 μM) or capsaicin (33 μM), to check for the development of tachyphylaxis or cross-tachyphylaxis, respectively.

The protocol was approved by the Animal Use Committee of the Karolinska Institute.

Pungency on the human tongue

Filter paper discs (1 cm in diameter) were impregnated with increasing amounts (starting at 0.05 μg) of the dialdehydes dissolved in acetone. After the solvent had evaporated, the discs were placed on the tip of the tongue of chemists at the Department of Organic Chemistry 2, University of Lund, Lund, Sweden. The amount of dialdehyde was increased in the discs until the sample became perceptibly pungent. To avoid the development of desensitization, the experiments were performed in such a way that at least 24 h elapsed between sampling two different compounds (Karrer & Bartoshuk, 1991).

Such unsaturated dialdehydes have been previously tested on the human tongue, and were found to be pungent but otherwise harmless (Kubo & Ganjian, 1981; Caprioli *et al.*, 1987; Cimino *et al.*, 1987).

According to the Swedish regulations, topical application of known non-harmful compounds on the tongue, when performed in informed volunteers (in our case, the chemists involved in the synthetic work), do not require approval of an Ethics Committee.

Inhibition of specific binding of [^{3}H]-resiniferatoxin

Membranes for binding experiments were prepared and binding assays with [^{3}H]-resiniferatoxin (37 Ci mmol^{-1}; Chemical Synthesis and Analysis Laboratory, NCI-FCRDC, Frederick, MD, U.S.A.) were performed according to published procedures (Szallasi *et al.*, 1992; 1993; Acs *et al.*, 1994). Briefly, female Sprague-Dawley rats (200–250 g) were killed by cervical dislocation and their trigeminal ganglia, along with the cervical segment of the spinal cord, were collected into ice-cold buffer A (pH 7.4), which contained (in mM) KCl 5, NaCl 5.8, $CaCl_2$ 0.75, $MgCl_2$ 2, sucrose 320, and HEPES 10. The tissues were disrupted in buffer A with the aid of a Polytron tissue homogenizer. The homogenates were first centrifuged for 10 min at 1,000 *g* (4°C); the low speed pellets were discarded; the supernatants were further centrifuged for 30 min at 35,000 *g* (4°C); and the resulting high speed pellets, resuspended in buffer A, were stored at −70°C until assayed.

Binding assays were carried out in a final volume of 500 μl, containing buffer A, supplemented with 0.25 mg ml^{-1} bovine serum albumin (Cohn fraction V; Sigma, St. Louis, MO, U.S.A.; included to stabilize ligands in aqueous solution), membranes (40–50 μg protein), [^{3}H]-resiniferatoxin (RTX), and non-radioactive ligands.

For determination of saturation curves for binding, membranes were incubated in triplicate with increasing concentrations of [^{3}H]-RTX (6–400 pM); non-specific binding was defined as that occurring in the presence of 100 nM non-radioactive RTX (LC Laboratories, Woburn, MA, U.S.A.). Binding was also analyzed in the presence of a fixed concentration of [^{3}H]-RTX (corresponding to the approximate K_d value from the saturation binding experiments) and various concentrations of competing ligands.

Assay mixtures were set up on ice and the binding reaction was then initiated by transferring the assay tubes to a 37°C water bath. Following a 60 min incubation period, the binding reaction was terminated by cooling the mixtures on ice, and then 100 μg of bovine α_1-acid glycoprotein (Sigma) was added to each tube to reduce non-specific binding (Szallasi *et al.*, 1992). At 0°C, the dissociation of specifically bound [^{3}H]-RTX is unmeasurably slow (Szallasi & Blumberg, 1993a); α_1-acid glycoprotein, however, readily binds free [^{3}H]-RTX (Szallasi *et al.*, 1992). Since free and non-specifically bound [^{3}H]-RTX are in equilibrium, α_1-acid glycoprotein by sequestering unbound RTX redistributes the non-specifically bound radioligand from the lipid phase to the aqueous phase and thereby reduces non-specific binding. Finally, bound and free [^{3}H]-RTX were separated by pelleting the membranes in a Beckman 12 microfuge (maximal velocity, 15 min). The [^{3}H]-RTX in the pellets and in aliquots of the supernatants were quantified by scintillation counting. Binding parameters were either determined by use of the curvilinear regression program LIGAND (Biosoft, Cambridge, U.K.), or analyzed by the computer program FitP (Biosoft), which fits the allosteric Hill equation to the measured values as described previously (Szallasi *et al.*, 1993; Acs *et al.*, 1994).

Isovelleral-induced $^{45}Ca^{2+}$-uptake by rat dorsal root ganglion neurones

The spinal columns of adult Sprague-Dawley rats were removed aseptically and dorsal root ganglia (DRG) from all levels were dissected out and collected in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g l^{-1} glucose, 0.5% heat inactivated foetal bovine serum (Gibco BRL, Gaithersburg, MD, U.S.A.), 1 mM Na-pyruvate, 25 mM HEPES and antibiotics (Wood *et al.*, 1988; Acs *et al.*, 1995). Ganglia were digested twice with 0.125% collagenase (Sigma) in DMEM for 90 min at 37°C. After being washed with DMEM containing 1 mM Na-pyruvate and 25 mM HEPES, ganglia were triturated through a flame polished Pasteur pipette to form a single cell suspension. Following removal of the myelin debris by pelleting the cells through a cushion of DMEM containing 15% fatty acid free bovine serum albumin (Sigma), cells were plated in Multiscreen-DV 96-well filtration plates (Millipore, Marlborough, MA, U.S.A.) at an approximate density of 5,000 cells per well, and were used immediately for calcium uptake experiments.

DRG neurones were incubated in DMEM containing 1.8 mM $CaCl_2$ in the presence of 0.25 mg ml^{-1} bovine serum albumin and 1 μCi ml^{-1} $^{45}Ca^{2+}$ (23.55 mCi mg^{-1}; DuPont-New England Nuclear, Boston, MA, U.S.A.) and various concentrations of isovelleral (25–3200 nM) for 20 min at 37°C. $^{45}Ca^{2+}$ influx by 500 nM isovelleral, a concentration giving maximal response in the above experiments, was also analyzed in the presence of 10 μM capsazepine (RBI, Natick, MA, U.S.A.). Cells were then washed six times with ice-cold

serum-free DMEM by use of a MultiScreen Vacuum Manifold (Millipore). Filters were dried under a heat lamp, and the radioactivity was determined by scintillation counting. For each data point 8 wells were analysed, and dose-response curves for stimulation were fitted by computer to the Hill equation (Acs *et al.*, 1995).

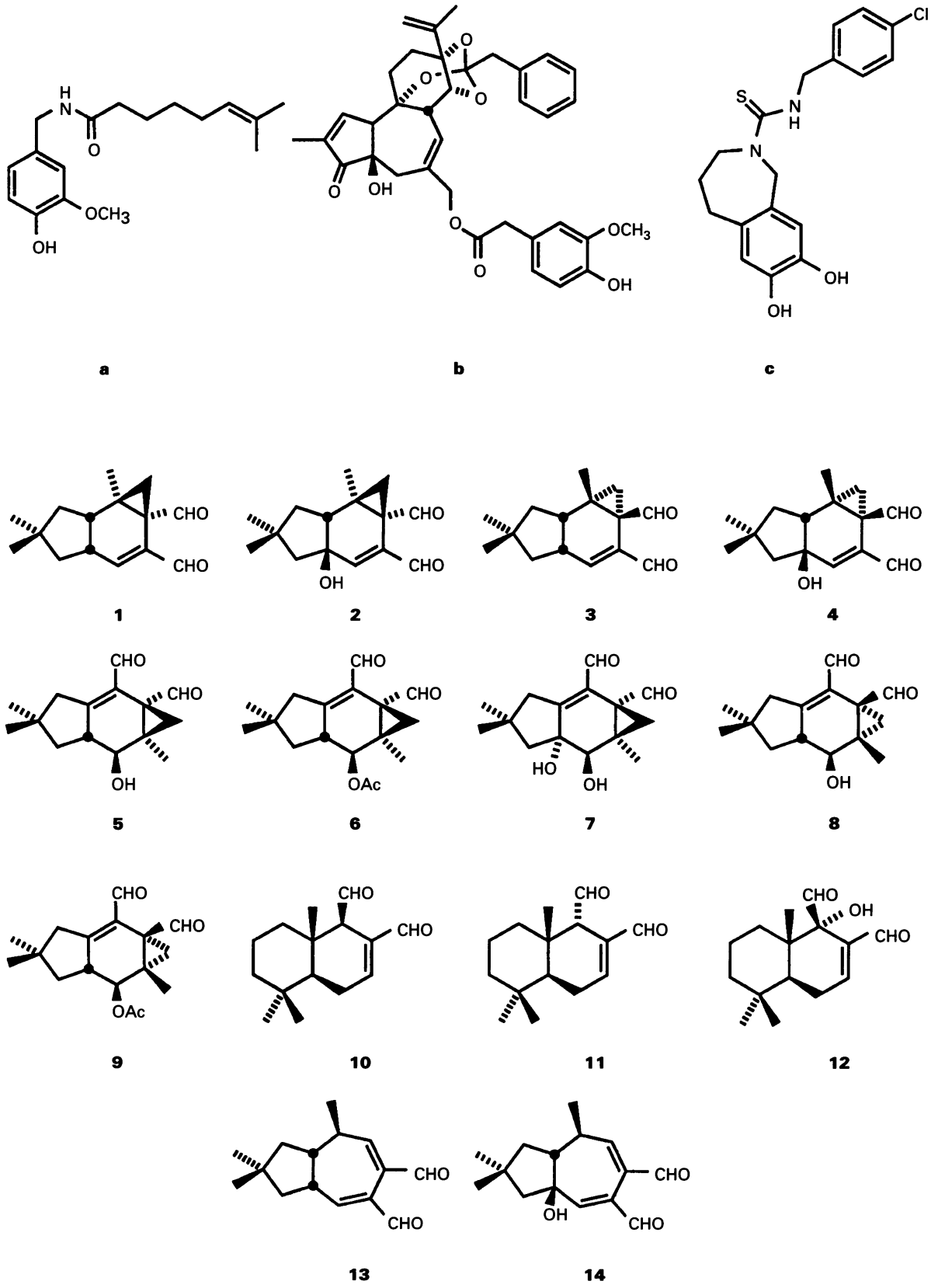

Figure 1 The structures of the unsaturated dialdehydes assayed in this study. All compounds are pure enantiomers, and the absolute stereochemistries are as shown. (1) Isovelleral; (2) 9-βOH-isovelleral; (3) isoisovelleral; (4) 9-βOH-isoisovelleral; (5) merulidial; (6) acetylmerulidial; (7) 9-αOH-merulidial; (8) isomerulidial; (9) acetylisomerulidial; (10) polygodial; (11) epipolygodial; (12) warburganal; (13) velleral; (14) 9-βOH-velleral. The structures of typical vanilloids such as capsaicin (a) and resiniferatoxin (b), as well as the vanilloid receptor antagonist capsazepine (c), are also shown for comparison.

Drugs and chemicals

(+)-Isovelleral (M.W. 232) and (−)-velleral (M.W. 232) were isolated from fruit bodies of *Lactarius vellereus*, according to a published procedure (Sterner *et al.*, 1985). (−)-Merulidial (M.W. 248) was extracted from submerged cultures of the fungus *Merulius tremellosus*, by use of the procedure of Quack and coworkers (1978), whereas (−)-polygodial (M.W. 234) was purified from the plant *Polygonum hydropiper*, as described by Tozyo and colleagues (1992). 9-β-hydroxyisovelleral (M.W. 248), 9-β-hydroxymerulidial (M.W. 264), and 9-β-hydroxyvelleral (M.W. 248) were prepared by autooxidation of their parent compounds (Sterner *et al.*, 1985; Sterner & Steglich, 1988). The isomers, isoisovelleral and 9-β-hydroxyisoisovelleral, were prepared from their parent compounds by thermal rearrangements in refluxing mesitylene for 60 min, followed by chromatographic separation. Acetylmerulidial (M.W. 290) as well as isomerulidial were obtained from merulidial as described previously (Giannetti *et al.*, 1986; Jonassohn *et al.*, 1995). Epipolygodial was prepared by epimerization of polygodial at 55°C in tetrahydrofuran (THF) with Cs_2CO_3 for 2 h. Finally, warburganal (M.W. 250) was obtained semisynthetically from natural (−)-polygodial, by reduction of the diol with excess $LiAlH_4$ in dry Et_2O, and subsequent oxidation (Hollinshead *et al.*, 1983; Urones *et al.*, 1994). All the other chemicals including capsaicin were from Sigma (St. Louis, MO, U.S.A.) unless indicated otherwise, and were of the highest quality available.

Results

Pungency of isovelleral in the rat eye

Isovelleral (structure shown in Figure 1) instilled into the eyes of rats became perceptible at concentrations higher than 4 μM, as measured in the eye-wiping assay. At a concentration of 400 μM, it reproducibly induced a response of 10 wipings or more (not shown). This response was similar to that induced by 33 μM capsaicin (see structure in Figure 1) solution. However, to avoid unnecessary discomfort to the animals, full dose-response curves were not determined. Six hours later, a second administration of isovelleral did not result in any wiping movements, indicating the development of tachyphylaxis; the same animals did not react to capsaicin either, or showed an attenuated response (occasional wipings) only. The lack of response to capsaicin in isovelleral-treated rats is indicative of cross-tachyphylaxis between these two irritant compounds, which was confirmed by the inability of isovelleral to provoke eye-wiping movements in rats desensitized to capsaicin topically (not shown).

Isovelleral-induced $^{45}Ca^{2+}$ uptake by rat cultured DRG neurones

Isovelleral induced a saturable $^{45}Ca^{2+}$ influx response in rat isolated DRG neurones cultured *in vitro* in a dose-dependent manner (Figure 2). Half-maximal response occurred at a concentration of 95±7 nM (mean±s.e.mean; 4 determinations; Figure 2). The maximal $^{45}Ca^{2+}$ uptake induced by 500 nM isovelleral was reduced in the presence of 10 μM capsazepine by 93±2% (significant at the level of $P<0.001$, Student's t test; mean±s.e.mean; 3 determinations; Figure 3).

Inhibition by isovelleral of specific [^{3}H]-resiniferatoxin binding

Isovelleral inhibited [^{3}H]-resiniferatoxin (RTX) (see Figure 1 for structure) binding to rat trigeminal ganglion (not shown) or spinal cord membranes (Figure 4) with similar IC_{50} values of 17.2±4.3 μM and 21.6±4.3 μM, respectively (mean± s.e.mean; 3 determinations). If membranes were pre-incubated for 30 min at 37°C in the presence of isovelleral before addition of [^{3}H]-RTX, the apparent affinity both for trigeminal ganglia and spinal cord membranes increased by 3–4 fold, yielding IC_{50} values of 7.8±1.3 μM and 5.2±0.9 μM, respectively (mean±s.e.mean; 4 determinations; Figure 4). In experiments in which the concentration of [^{3}H]-RTX was varied (Figure 5), 5 μM isovelleral, as expected, reduced the apparent affinity of RTX to specific binding sites in spinal cord from 16±2 pM to 37±4 pM, without having any measurable effect on B_{max} (112±6 fmol mg^{-1} protein in the absence and 117±15 fmol mg^{-1} protein in the presence of isovelleral, respectively). Unexpectedly, isovelleral also changed the Hill coefficient from 2.1±0.1 to 1.5±0.1 (mean±s.e.mean; $n=3$);

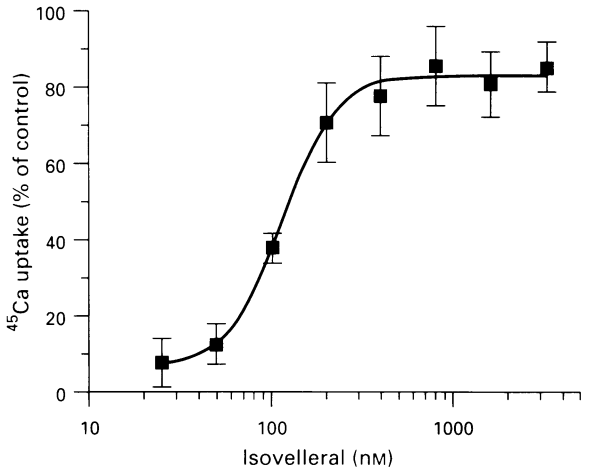

Figure 2 Isovelleral-induced $^{45}Ca^{2+}$-uptake by adult rat dorsal root ganglia neurones cultured *in vitro*. Points represent the mean values from 4 experiments; error bars indicate s.e.mean. In each experiment, 3–5 determinations were made for each point. For each experiment, basal ^{45}Ca influx was subtracted and values were calculated as % of ^{45}Ca influx induced by 3 μM capsaicin. The mean basal ^{45}Ca influx was 342±26 d.p.m./well; that for capsaicin-induced ^{45}Ca influx was 557±20 d.p.m./well.

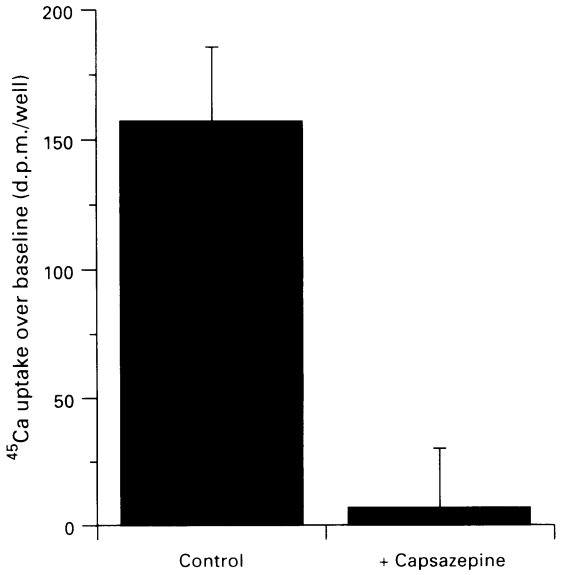

Figure 3 $^{45}Ca^{2+}$-uptake over baseline by rat dorsal root ganglia neurones induced by 500 nM isovelleral in the absence (control) or presence of 10 μM capsazepine. Error bars indicate s.e.mean of triplicate determinations. Two additional experiments gave similar results. Inhibition by capsazepine was significant at $P<0.001$; Student's t test.

that is, it reduced the positive co-operativity that characterizes RTX binding (Figure 5).

Pungency of terpenoids on the human tongue; correlation with their potency to inhibit [^{3}H]-resiniferatoxin binding to rat spinal cord membranes

Isovelleral produced a distinct burning sensation on the human tongue at a dose of 2.2 nmol (Table 1; Figure 6). Out of the 14 natural as well as semisynthetic terpenoids tested (structures shown in Figure 1), 4 turned out to inhibit RTX binding with marginal potency, i.e. with less than 50% inhibition at the highest concentration tested (100 μM). These compounds were at least 10 fold less irritant than isovelleral on the human tongue (Table 1). One compound, namely polygodial, was more pungent on the human tongue (0.4 nmol) than expected based on its potency ($IC_{50} = 7.6$ μM) for specific RTX binding sites in rat spinal cord membranes (Table 1). The other nine compounds showed a good correlation ($r = 0.76$; $P < 0.000001$, Multiple Regression test) between pungency and binding affinity (Table 1; Figure 6).

Discussion

The present study was designed to evaluate the possibility that naturally occurring pungent terpenoids such as isovelleral, possessing an unsaturated 1,4-dialdehyde moiety, exert a

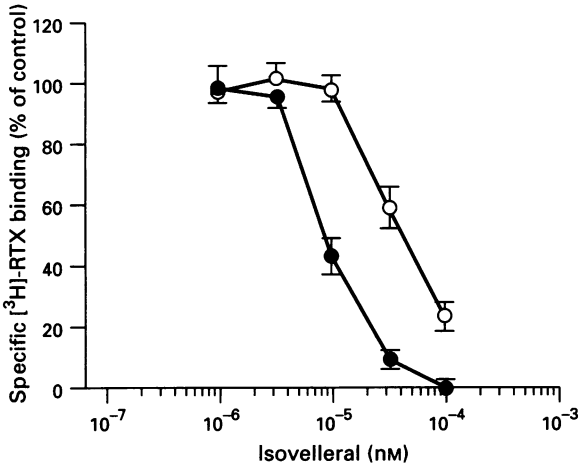

Figure 4 Inhibition by isovelleral of specific [^{3}H]-resiniferatoxin (RTX) binding to rat spinal cord membranes when ligands were added together (○) or isovelleral was employed in a 30 min preincubation protocol (●). Isovelleral inhibited RTX binding with an approximately 4 fold greater potency when preincubated for 30 min before the addition of RTX. Points represent mean values from a single experiment ± s.e.mean (vertical lines); 3 additional experiments gave similar results.

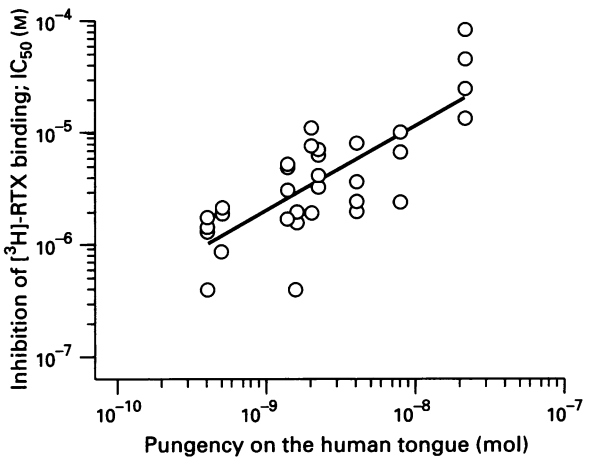

Figure 6 Correlation between the pungency of 9 unsaturated dialdehydes on the human tongue and their ability to inhibit specific [^{3}H]-resiniferatoxin (RTX) binding to rat spinal cord membranes. Data are from Table 1 (compounds 1–6, and 11–13). Points represent individual binding affinity measurements, used to calculate the mean ± s.e.mean. values in Table 1. Multiple regression analysis suggests that this correlation is highly significant (correlation coefficient 0.76; $P < 0.000001$).

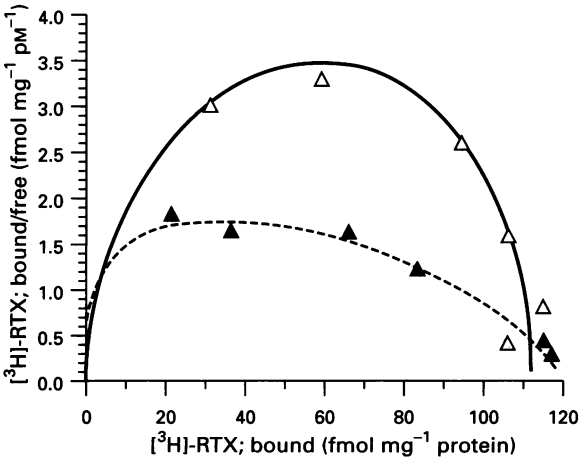

Figure 5 Scatchard plots of specific [^{3}H]-resiniferatoxin (RTX) binding to rat spinal cord membranes in the absence (△) or presence (▲) of 5 μM isovelleral. Both Scatchard plots are convex, consistent with positive binding co-operativity; however, isovelleral reduced the curvature of the Scatchard plot. As shown, co-operativity indices are 2.1 and 1.4 in the absence and presence of isovelleral, respectively. Note that isovelleral diminished the apparent affinity for RTX (from 16 pM to 37 pM), but did not change the B_{max}.

Table 1 Pungency of natural as well as semisynthetic terpenoids with an unsaturated 1,4-dialdehyde moiety on the human tongue, and their affinity for specific [^{3}H]-resiniferatoxin binding sites in rat spinal cord preparations

Compound	*Pungency* (nmol/tongue)	*Affinity* (IC_{50}; μM)
Isovelleral (1)	2.2	5.2 ± 0.9
9-βOH-isovelleral (2)	8.1	6.6 ± 2.0
Isoisovelleral (3)	0.4	1.3 ± 0.4
9-βOH-isoisovelleral (4)	4.0	4.0 ± 1.2
Merulidial (5)	1.6	1.2 ± 0.4
Acetylmerulidial (6)	1.4	3.8 ± 0.7
9-αOH-merulidial (7)	19.0	> 100
Isomerulidial (8)	80.6	> 100
Acetylisomerulidial (9)	69.0	> 100
Polygodial (10)	0.4	7.6 ± 0.9
Epipolygodial (11)	21.4	43.2 ± 17.1
Warburganal (12)	2.0	6.8 ± 2.4
Velleral (13)	0.5	1.7 ± 0.9
9-βOH-velleral (14)	20.2	> 100

The numbers in parentheses correspond to the numbers in Figure 1.

Note: at concentrations higher than 100 μM, an increase in the non-specific binding of [^{3}H]-resiniferatoxin was observed.

pungent effect by exciting capsaicin-sensitive sensory neurones in a vanilloid receptor-mediated fashion. Our findings argue cogently that this is the case. Consequently, these compound appear to represent a novel class of potent ligands acting on vanilloid receptors.

Structure-activity relationships for capsaicinoids have been explored in depth (Szolcsányi & Jancsó-Gábor, 1975; 1976; Hayes *et al.*, 1984; Janusz *et al.*, 1993; Walpole & Wrigglesworth, 1993), and there is a consensus in the literature that an intact (homo)vanillyl moiety is critical for biological activity. The discovery of ultrapotent capsaicin analogues, as exemplified by RTX, although shedding new light on the role of the 'side-chain' in compounds with capsaicin-like activity, confirmed the importance of the homovanillyl substituent (Szallasi & Blumberg, 1990; Blumberg *et al.*, 1993). In keeping with this, the recognition site for these irritant compounds was termed the vanilloid receptor (Szallasi & Blumberg, 1990b; Szallasi, 1994). Given the strict structure-activity requirements for vanilloid-like activity, it is intriguing that terpenoids lacking any homovanillyl-like substituent act on vanilloid receptors.

A great variety of xenobiotics activate capsaicin-sensitive nerves (cf Maggi, 1991; Rang *et al.*, 1994), and some of these xenobiotics, such as the alkylating agent N-ethylmaleimide (Evangelista *et al.*, 1992) or the heavy metal cadmium (Dray *et al.*, 1990) and nickel (Wood *et al.*, 1988), also interfere with specific [^{3}H]-RTX binding (Szallasi & Blumberg, 1993a). Since these agents are known to react with free sulphydryl groups, it was suggested that the vanilloid receptor is a thiol-protein (Szallasi & Blumberg, 1993a). It is, however, by no means certain that such sulphydryl-reactive agents excite capsaicin-sensitive nerves by activating vanilloid receptors. In the case of $CdCl_2$, the apparent inability of capsazepine to block the activation of the rat urinary bladder (R. Patacchini, personal communication) clearly argues against the involvement of vanilloid receptors. Therefore, it is not clear whether or not the reactivity of unsaturated dialdehydes toward thiol groups (Kubo & Ganjian, 1981; Cimino *et al.*, 1987) plays any role in their interaction at vanilloid receptors. The 14 terpenoids tested in this study, although all of them contain an unsaturated 1,4-dialdehyde functionality, show considerable differences in pungency (for example, isoisovelleral is 200 fold more active than isomerulidial), which also argues against a simple chemical reactivity as the sole underlying mechanism of action.

The mechanism by which pungent terpenoids bind to vanilloid receptors remains unclear. The interaction between vanilloids and their receptors is clearly complex. Binding to vanilloid receptors shows positive co-operativity (Szallasi *et al.*, 1993; Szallasi, 1994; Acs *et al.*, 1996), as does channel opening measured in outside-out patches (Oh *et al.*, 1996). On the other hand, calcium uptake in response to vanilloids was shown to be non-cooperative (Acs *et al.*, 1996), and certain ligands, e.g. phorbol 12-phenylacetate 13-acetate 20-homovanillate, reduce the cooperativity of RTX binding to 1 (Szallasi *et al.*, 1996). Isovelleral, similarly, reduces the co-operativity index of approximately 2 which characterizes RTX binding to rat spinal cord membranes (Szallasi *et al.*, 1993; Acs *et al.*, 1994), although it has no effect on B_{max}. The effect on co-operativity suggests that the binding of isovelleral is at least partly non-competitive. However, since homovanillyl group-containing vanilloids are likewise able to reduce binding co-operativity (Szallasi *et al.*, 1996), an effect on cooperativity does not necessarily mean non-competitive binding.

As regards the structure-activity relationships, the presence of a hydroxyl group at the bridgehead position of unsaturated dialdehydes appears to play an important role in that it consistently reduces activity (see Table 1). Most striking is the difference between merulidial, one of the most potent compounds both in assays for pungency and vanilloid receptor binding, and its 9-hydroxylated derivative, which is 50 fold less active. The stereochemistry of the compounds is also important. Again, a striking difference in activity is observed between merulidial and its isomer (isomerulidial). And last but not least, inversion of the cyclopropane ring also appeared to diminish activity. It has previously been suggested that the absolute configuration of the unsaturated dialdehyde moiety is important for bioactivity (Jonassohn *et al.*, 1995); in order to test this hypothesis, the synthesis of the enantiomers of the compounds described in this paper is in progress.

Vanilloids have a clear therapeutic potential to mitigate neuropathic pain and to alleviate symptoms in which mediators released from capsaicin-sensitive nerves play a major role (Lynn, 1990; Carter, 1991; Maggi, 1992; Szallasi & Blumberg, 1993b; Winter *et al.*, 1995). The clinical use of vanilloids is, however, severely limited by their irritant effect, and the development of novel vanilloids, devoid of such side-effects, is therefore of considerable interest. Until now, it was generally accepted that vanilloid-like activity requires the presence of a homovanillyl substituent (Szolcsányi & Jancsó-Gábor, 1975; Walpole & Wrigglesworth, 1993). The present study, by showing that a family of natural irritant compounds structurally unrelated to capsaicin/RTX-like ligands can activate capsaicin-sensitive neurones in a vanilloid receptor-mediated fashion, may provide important new clues to our understanding of how vanilloid receptors recognize ligands, and thus can ultimately lead to the rational design of novel, innovative 'vanilloids'. Of course, unsaturated dialdehydes are not real vanilloids from a chemical point of view. Receptors are preferentially named after their endogenous activators. Hopefully, such endogenous activators of 'vanilloid' receptors will shortly be identified and thus the proper taxonomy for 'vanilloid' receptors and their ligands resolved.

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