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Dialdehyde sesquiterpenes and other terpenoids as vanilloids

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Abstract

Selected naturally occurring unsaturated dialdehyde sesquiterpenes and related bioactive terpenoids were assayed for vanilloid-like activity. Out of the 25 compounds tested, eight inhibited completely the specific binding of [^{3}H]resiniferatoxin by rat spinal cord membranes: binding affinities ranged from 0.6 μM for cinnamodial to 19.0 μM for hebelomic acid F. These values were comparable to the binding affinity of capsaicin (2.7 μM). With the exception of four ligands, compounds that inhibited resiniferatoxin binding to rat spinal cord membranes were also pungent on the human tongue where they showed cross-tachyphylaxis with capsaicin. As expected from their reactive nature, these compounds possess additional sites of action, as reflected in the complex behavior of the stimulation of calcium influx by cinnamodial and cinnamosmolide at high concentrations. This observation might explain the unexpectedly weak membrane depolarization by cinnamodial compared to capsaicin. We conclude that a range of sesquiterpene dialdehydes and related terpenoids, both pungent and non-pungent, may function as vanilloids. These compounds may represent a new chemical lead for the development of vanilloid drugs, structurally unrelated to either capsaicin or resiniferatoxin.

Keywords: Vanilloid receptor; Resiniferatoxin; Capsaicin; Dialdehyde, unsaturated

1. Introduction

In response to evolutionary pressure, many organisms have developed chemical defenses. In particular, to date more than 80 terpenoid unsaturated 1,4-dialdehydes, mainly sesquiterpenes, have been identified (Jonassohn and Sterner, 1997). They are found in plants, fungi, insects and other animals (Jonassohn and Sterner, 1997). Typically, terpenoid dialdehydes are very versatile repellents. Their described bioactivities range from antimicrobial (Anke and Sterner, 1991) to antifeedant (Camazine et al., 1983) to toxic (Forsby et al., 1991) in order to deter insects, worms, and herbivores at the same time. This broad range of activities predicts an array of biological targets for sesquiterpene dialdehydes and related terpenoids (Jonassohn and Sterner, 1997).

Biological pathways activated by sesquiterpene dialdehydes and other bioactive terpenoids may provide important clues to identify such targets. For instance, the majority of terpenoids containing an unsaturated 1,4-dialdehyde functionality are intensely pungent (Kubo and Ganjian, 1981). The extract of the bark of *Cinnamosma fragrans*, a native plant of Madagascar, which contains several sesquiterpenes (e.g., cinnamolide, cinnamodial, and cinnamosmolide), was described as having 'a distinct pepper-like taste' (Canonica et al., 1969). As a matter of fact, other plant sources of pungent dialdehydes (for instance, water pepper, the source of polygodial) are used as spices to flavor food (Fukuyama et al., 1982).

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Pepper-like substances such as capsaicin, the active ingredient in red pepper, as well as piperone, responsible for the piquancy of black pepper, are pungent by activating a common membrane recognition site on sensory neurons (Jancsó, 1968; Szolcsányi and Jancsó-Gábor, 1975; Szallasi and Blumberg, 1990), referred to as the vanilloid receptor (Szallasi and Blumberg, 1996). As predicted by the similar 'hot' sensation evoked by capsaicin (Rozin and Schiller, 1980) and unsaturated dialdehydes (Kubo and Ganjian, 1981) on the human tongue, terpenoids were found to be pungent by activating capsaicin-sensitive neurons in a vanilloid receptor-mediated fashion (Szallasi et al., 1996).

The present study was undertaken to identify further vanilloids in a collection of naturally occurring sesquiterpenoid dialdehydes and related bioactive terpenoids. For a better comparison with previous studies (Acs et al., 1996; Szallasi et al., 1996), the assays of $^{45}Ca^{2+}$ uptake by cultured rat dorsal root ganglion neurons and [^{3}H]resiniferatoxin binding to rat spinal cord preparations were chosen to characterize terpenoid interactions at vanilloid receptors. As an in vivo measure of potency, the pungency of terpenoids on the human tongue was determined (Kubo and Ganjian, 1981; Szallasi et al., 1996). Finally, one compound, cinnamodial, was also administered to cultured sensory neurons under current-clamp conditions in order to characterize its activity at the electrophysiological level.

2. Materials and methods

2.1. Pungency on the human tongue

Filter paper disks (measuring 1 cm in diameter) impregnated with increasing amounts of sesquiterpene dialdehydes and other terpenoids (starting at 0.2 nmol) were placed on the tip of the tongue of volunteers until pungency was perceived, as described in detail previously (Szallasi et al., 1996). To avoid the development of desensitization, at least 24 h were allowed to elapse between sampling two different compounds (Karrer and Bartoshuk, 1991). To examine cross-desensitization between capsaicin and isovelleral, three doses of capsaicin (1 nmol each) were applied on the tongue at 2 h intervals. Desensitization to capsaicin was confirmed by the lack of pungency to a subsequent capsaicin application. Cross-desensitization was evaluated by exposing the tongues to a dose of 2.2 nmol isovelleral, the EC_{50} value from prior pungency measurements (Szallasi et al., 1996).

The volunteers participating in these experiments were chemists involved in the synthetic work at the Universities of Pavia, Italy, and Lund, Sweden. According to Swedish and Italian regulations, topical application of non-harmful compounds to informed volunteers does not require approval of an Ethics Committee.

2.2. [^{3}H]resiniferatoxin binding to rat spinal cord membranes

Binding studies with [^{3}H]resiniferatoxin were carried out according to a published protocol in which non-specific resiniferatoxin binding is reduced by adding bovine α_1-acid glycoprotein after the binding reaction has been terminated (Szallasi and Blumberg, 1992). To obtain membranes for the binding studies, female Sprague–Dawley rats weighing 200–250 g were euthanized under CO_2 anesthesia, the cervical segment of the spinal cord was removed and disrupted with the aid of a tissue homogenizer in an ice-cold buffer (pH 7.4) containing (in mM) KCl 5, NaCl 5.8, $CaCl_2$ 0.75, $MgCl_2$ 2, sucrose 320, and HEPES 10. Spinal cord homogenates were first centrifuged for 10 min at $1000 \times g$ (4°C) to remove the nuclear fraction and debris and then the supernatant from the first centrifugation was further centrifuged (30 min; $35{,}000 \times g$; 4°C) to obtain a partially purified membrane fraction. Membranes resuspended in the homogenization buffer were stored at −70°C until assayed.

Competition binding assays were performed in a final volume of 500 μl, containing buffer (same as homogenization buffer) supplemented with 0.25 mg/ml bovine serum albumin, 40–50 μg of membrane protein, 20 pM [^{3}H]resiniferatoxin (the approximate K_d value from previous saturation experiments), 100 nM non-radioactive resiniferatoxin to determine non-specific binding, and increasing concentrations (up to 100 μM) of unsaturated dialdehydes.

Since resiniferatoxin binding shows marked temperature-dependence (at 0°C both association and dissociation are unmeasurably slow), assay mixtures were set up on ice and then the binding reaction was initiated by transferring the assay tubes to a 37°C shaking water bath. Following a 60 min incubation period, the binding reaction was terminated by cooling the mixtures on ice. To reduce non-specific binding, 100 μg of bovine α_1-acid glycoprotein was added to each tube; unlike vanilloid receptors, this serum protein readily binds free resiniferatoxin at 0°C (Szallasi et al., 1992). Since non-specific resiniferatoxin binding to membranes is in equilibrium with the unbound resiniferatoxin concentration in the aqueous phase, bovine α_1-acid glycoprotein is able to reduce non-specific binding by sequestering unbound [^{3}H]resiniferatoxin (Szallasi et al., 1992). Receptor binding is, however, not compromised as there is no detectable dissociation from the receptor at 0°C.

Finally, bound and free [^{3}H]resiniferatoxin were separated by pelleting the membranes in a Beckman 12 benchtop centrifuge (maximal velocity; 15 min; 4°C), and radioactivity was determined by scintillation counting. Binding parameters were determined by the curvilinear regression program of Munson and Rodbard (LIGAND, Biosoft, Ferguson, MO). Alternatively, measured values were fitted to the allosteric Hill equation with the aid of the computer program FitP (Biosoft, Ferguson, MO) as described previ-

ously (Szallasi et al., 1993). Values for binding affinities are presented as the mean ± S.E.M. of at least three determinations. In each experiment, values at each ligand concentration were determined in triplicate.

2.3. Calcium isotope uptake by cultured rat dorsal root ganglion neurons

Dorsal root ganglia were collected from Sprague–Dawley rats (females, weighing 200–250 g) under aseptic conditions and digested twice with 0.125% collagenase in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with HEPES and antibiotics, for 90 min at 37°C (Acs et al., 1996). Digested ganglia were triturated through a flame-polished Pasteur pipette to form a single-cell suspension; the myelin debris was removed by pelleting the cells through a cushion of DMEM containing 15% fatty acid free bovine serum albumin; and then the cells were plated in Multiscreen-DV 96-well filtration plates at an approximate density of 5000 cells per well. Cells were used for calcium isotope uptake experiments immediately after plating.

Dorsal root ganglion neurons were incubated in DMEM containing 1.8 mM $CaCl_2$ in the presence of 0.25 mg/ml bovine serum albumin, 1 μCi/ml $^{45}Ca^{2+}$, and various concentrations of dialdehydes for 30 min at 37°C. Maximal $^{45}Ca^{2+}$ uptake response by dialdehydes was also analyzed in the presence of the vanilloid receptor antagonist capsazepine (10 μM) (Bevan et al., 1992).

Calcium uptake was terminated by washing the cells six times with ice-cold DMEM with the aid of a Multiscreen Vacuum Manifold (Millipore, Marlborough, MA); filters were dried under a heat lamp; and the radioactivity was determined by scintillation counting.

2.4. Current-clamp analysis of cinnamodial-evoked responses

Dorsal root ganglia were excised from adult male Sprague–Dawley rats (180–220 g). Neurons were isolated enzymatically as described (Petersen et al., 1996), plated on poly-L-lysine coated glass coverslips, and maintained in Ham's F12 culture medium supplemented with heat-inactivated horse serum (10%), nerve growth factor 7S (100 ng/ml), L-glutamine (2 mM), and antibiotics until assayed.

Recordings were performed 6 to 36 h after plating with the aid of an Axopatch 200A (Axon Instrument, Foster City, CA) amplifier set to whole-cell patch-clamp method in current-clamp mode. A coverslip with the cells was placed in a recording chamber filled with 0.5 ml of a solution containing (in mM) NaCl 140, KCl 5, $CaCl_2$ 2, and $MgCl_2$ 1. The recording electrode was filled with a buffer (pH 7.3) that contained (in mM) KCl 140, $CaCl_2$ 1, EGTA 11, HEPES 10, and Mg-ATP 2, and had a final resistance in the range of 2 to 6 MΩ.

Before cinnamodial application, the smallest amount of current (0.3, 0.5, or 1.0 nA for 500 ms) that elicited an action potential was determined. The depolarizing threshold current was repeatedly applied every 2 s for 500 ms during application of 10 μM cinnamodial for 1 min. Consequently, in every recording, the first action potential was elicited by the current injection, whereas the subsequent action potentials were generated by the drug.

Current command protocols and data acquisition were carried out using the pClamp6 software (Axon Instruments, Foster City, CA).

2.5. Materials

[^{3}H]Resiniferatoxin (37 Ci/mmol) was synthesized by the Chemical Synthesis and Analysis Laboratory, NCI-FCRDC, Frederick, MD. $^{45}Ca^{2+}$ (23.55 mCi/mg) was purchased from DuPont-NEN, Boston, MA. Non-radioactive resiniferatoxin was obtained from Alexis Corp. (San Diego, CA) and capsazepine was from RBI (Natick, MA).

The isolation of natural (+)-isovelleral from fruit bodies of *Lactarius vellereus*, as well as the preparation of (−)-isovelleral, (−)-isoisovelleral, and (+)-isoisovelleral were carried out as described (Jonassohn et al., 1997). Aframodial, isolated from the seeds of *Aframomum daniellii*, was a kind gift from Prof. J.F. Ayafor, University of Yaounde, Cameroon. Scalaradial and desacetylscalaradial were isolated from the sponge *Cacospongia mollior* (De Rosa et al., 1994), and isocopalendial was obtained from *Spongia officinalis* (Cimino et al., 1982). Drimenol, as well as uvidin A and B were isolated from *Lactarius uvidus* (De Bernardi et al., 1980). Fasciculol B and C, along with their depsipeptide derivatives, were obtained from *Naematoloma sublateritium* (De Bernardi et al., 1981). Hebelomic acid A, B, and F were from *Hebeloma senescens* (Garlaschelli et al., 1995). Fuliginophenol was isolated from *Lactarius fuliginosus* and *Lactarius picinus* (De Bernardi et al., 1992). Ancistrodial, originally isolated from the defensive secretion of minor soldiers of the West African termite *Ancistrotermes cavithorax*, was prepared by total synthesis (Vidari et al., 1996). Ziniolide was isolated from *Xanthium catharticum* (Cumanda et al., 1991). Psilostachyin C and damsin were obtained from *Franseria artemisioides* (Vidari et al., 1997). Lactaroscrobiculide A and B, along with lactarotropone were isolated from *Lactarius scrobiculatus* (Battaglia et al., 1980; Bosetti et al., 1989; De Bernardi et al., 1993). Lactarorufin A was from *Lactarius blennius* (Vidari et al., 1976). Cinnamodial and cinnamosmolide, naturally found in *Cinnamosma fragrans*, *Warburgia ugandensis*, and *Capsicodendron dinisii*, were prepared from uvidin A as described (Garlaschelli and Vidari, 1989; Garlaschelli et al., 1991). The purity of the compounds was determined by HPLC as described in the respective references.

All the other chemicals used were purchased from Sigma (St. Louis, MO) and were of the highest quality available.

3. Results

3.1. Inhibition by dialdehyde sesquiterpenes of [^{3}H]resiniferatoxin binding to rat spinal cord preparations

In order to identify further vanilloids and to delineate structural requirements for such activity, a collection of 25 naturally occurring sesquiterpene dialdehydes and related terpenoids were screened in a [^{3}H]resiniferatoxin binding assay employing rat spinal cord membranes. Out of the 25 compounds tested, eight ligands (namely, aframodial, cinnamodial, cinnamosmolide, desacetylscalaradial, drimenol, hebelomic acid F, isocopalendial, and scalaradial, see Fig. 1 for structures) provided full binding inhibition at a concentration of 100 μM. An additional 5 compounds (ancistrodial, hebelomic acid A and B, fasciculol C-depsipeptide, and ziniolide) yielded partial inhibition. The use of higher concentrations was prevented by an apparent increase in non-specific [^{3}H]resiniferatoxin binding. For compounds giving a full inhibition of resiniferatoxin binding, affinities were determined in competition experiments (Table 1): K_i values ranged from 0.6 ± 0.2 μM (cinnamodial; mean ± S.E.M.; $n = 5$) to 19.0 ± 4.3 μM (hebelomic acid F; mean ± S.E.M.; $n = 3$). Capsaicin competed for specific resiniferatoxin binding sites with a similar affinity (2.7 ± 0.5 μM; mean ± S.E.M.; $n = 3$).

To study the effect of stereochemistry on receptor recognition, the binding affinities of natural isovelleral and isoisovelleral were determined along with their synthetic stereoisomers (see Fig. 1 for structures and Table 1 for results).

Table 1
Inhibition by terpenoid dialdehydes and related compounds of specific [^{3}H]resiniferatoxin (RTX) binding to rat spinal cord membranes and their pungency on the human tongue

	Binding affinity (μM)	Pungency (nmol/tongue)
(+)-Isovelleral	2.7 ± 0.2	2.2
(−)-Isovelleral	2.4 ± 0.3	0.4
(−)-Isoisovelleral	1.3 ± 0.2	0.4
(+)-Isoisovelleral	0.9 ± 0.1	2.2
Aframodial	11.6 ± 2.4	2.0
Cinnamodial	0.6 ± 0.2	17
Cinnamosmolide	1.5 ± 0.6	100
Desacetylscalaradial	6.3 ± 1.8	> 100
Drimenol	13.2 ± 4.0	> 100
Isocopalendial	4.4 ± 1.3	50
Hebelomic acid F	19.0 ± 4.3	> 100
Scalaradial	3.2 ± 1.7	> 100

Partial inhibition of RTX binding at 100 μM: ancistrodial; hebelomic acid A and B; fasciculol C-depsipeptide; ziniolide.
Inactive in the RTX binding assay at 100 μM: damsin; fasciculol B and C; fuliginophenol; fasciculol B-depsipeptide; lactarorufin A; lactaroscrobiculide A and B; lactarotropone; psilostachyin C; uvidin A and B.
K_i, mean ± S.E.M.; at least three determinations for each compound.

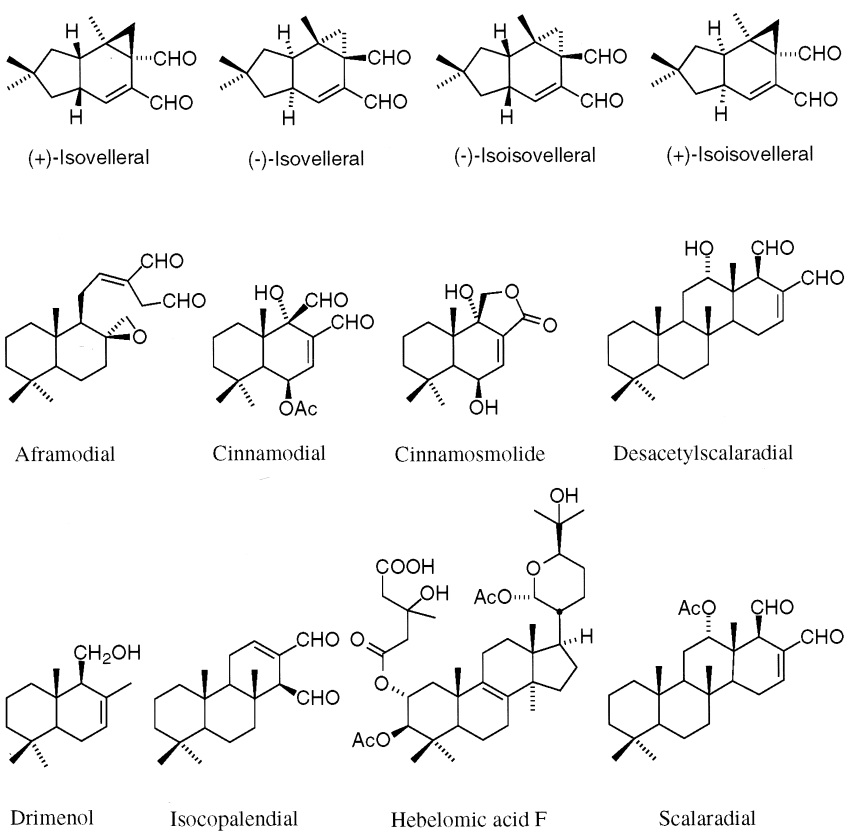

Fig. 1. Chemical structures of the sesquiterpene dialdehydes and related terpenoids examined in this study that fully inhibited [^{3}H]resiniferatoxin binding to rat spinal cord membranes.

3.2. Pungency of dialdehyde sesquiterpenes on the human tongue

A widely used rapid bioassay to study the potency of capsaicin congeners is based on their pungency on the human tongue. To verify that this approach is also applicable to unsaturated dialdehydes, isovelleral effects were compared on control tongues and on tongues desensitized to capsaicin. Isovelleral (2.2 nmol) evoked a burning, capsacin-like sensation on the human tongue which was completely abolished by prior capsaicin desensitization (see desensitization protocol in Section 2). Whereas stereoconfiguration had no (or only negligible) effects on the binding affinity of isovelleral and isoisovelleral, in the pungency of stereoisomers a moderate but clear (approximately 5-fold) difference was perceived.

As was reported previously and is confirmed in the present study, isovelleral evokes a 'hot', burning sensation on the human tongue, characteristic of capsaicin. For the sesquiterpenoid dialdehydes examined in the present study, a limited correlation was observed between the affinity of the compounds for specific resiniferatoxin binding sites in rat spinal cord membranes and their pungency on the human tongue (Table 1). For instance, cinnamodial was 4-fold more potent than isovelleral for binding but approximately 10-fold less pungent than isovelleral (Table 1). Furthermore, scalaradial was similar to isovelleral in binding affinity but was practically inactive in the assay of pungency (Table 1). Desacetylscalaradial has previously been reported to be more pungent than scalaradial, but compared to isovelleral, for example, it is much less pungent.

It is interesting to note that some related terpenoids lacking an unsaturated dialdehyde functionality, e.g., cinnamosmolide, drimenol, and hebelomic acid F, also have affinity for specific resiniferatoxin binding sites in rat spinal cord membranes, although these ligands are apparently marginally (cinnamosmolide) or not at all pungent on the human tongue (Table 1). Interestingly, these compounds evoke a bitter aftertaste.

3.3. Dialdehyde sesquiterpene-evoked calcium uptake by cultured rat dorsal root ganglion neurons

Cinnamodial, a compound combining stuctural features of unsaturated dialdehydes and cinnamosmolide (see Fig. 1 for structures), is both pungent and bitter tasting. These

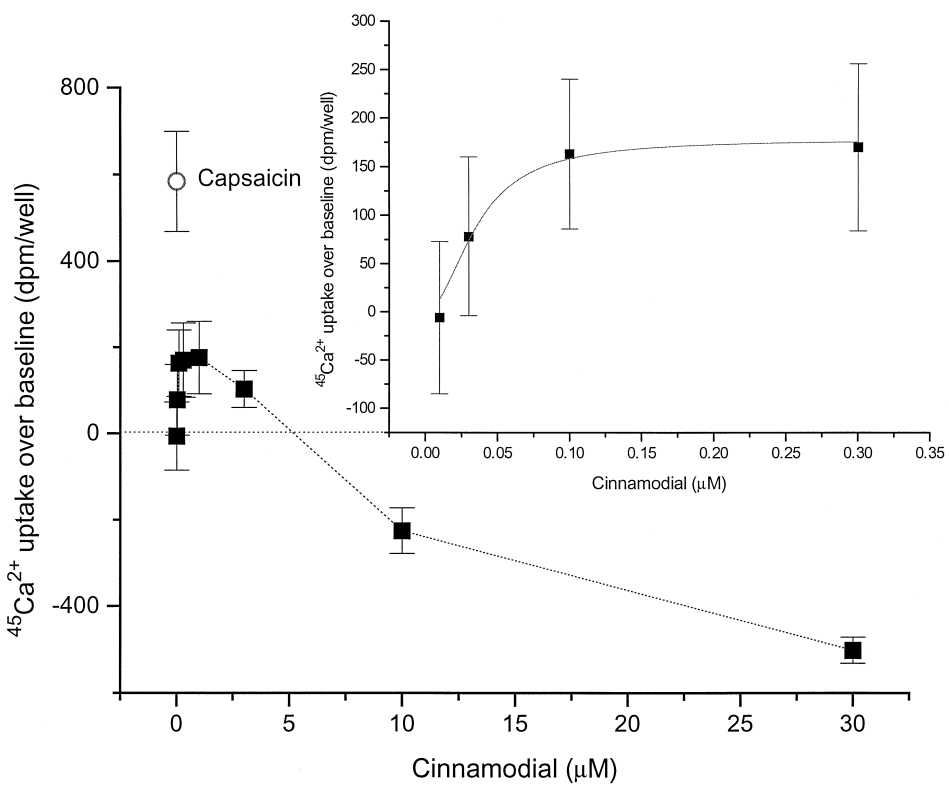

Fig. 2. Cinnamodial-induced $^{45}Ca^{2+}$-uptake by adult rat dorsal root ganglia neurons cultured in vitro. The figure depicts a representative experiment: points are mean values from a single determination performed in quintuplicate; error bars indicate ± S.E.M. Five additional experiments yielded similar results. Figure insert shows that the dose–response curve is, in fact, bi-phasic. The initial increase in calcium uptake is superseded by an inhibitory phase at higher cinnamodial concentrations which even reduces the baseline calcium uptake value. Note that due to the bi-phasic nature of the dose–response curve, cinnamodial acts as a partial vanilloid agonist: the maximal mean calcium uptake response by cinnamodial was 47 ± 9% of that evoked by capsaicin.

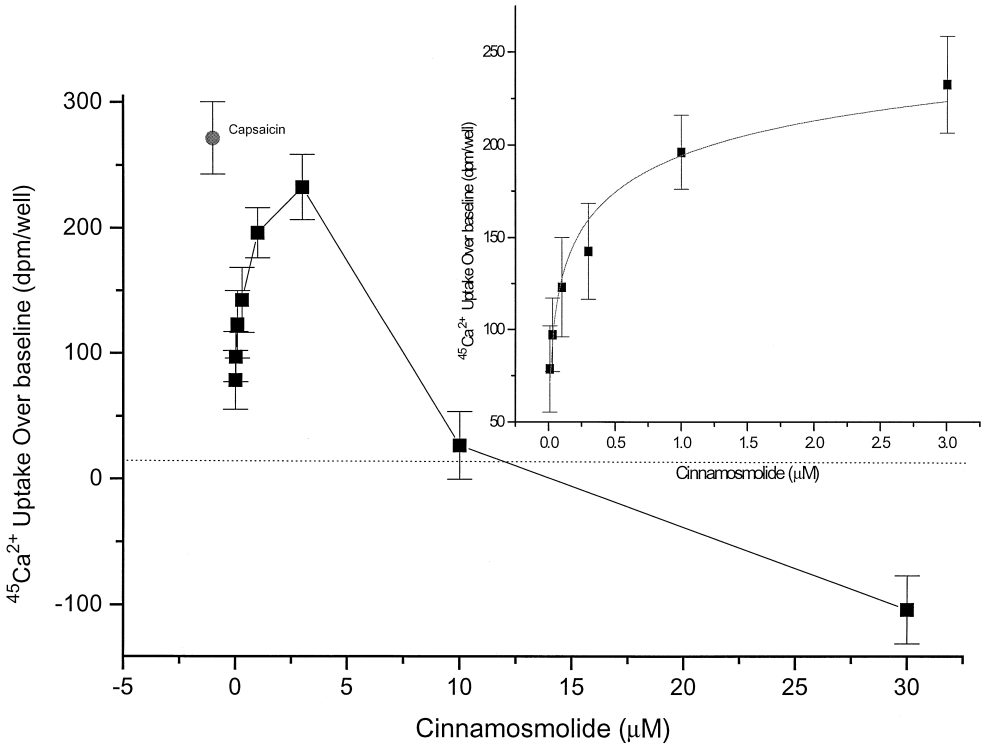

Fig. 3. Cinnamosmolide-evoked $^{45}Ca^{2+}$-uptake by adult rat dorsal root ganglia neurons cultured in vitro. Note that the dose–response curve is bi-phasic. At concentrations lower than 10 μM, cinnamosmolide induces a dose-dependent increase in calcium influx that reaches the maximal calcium uptake response by capsaicin. At concentrations higher than 10 μM, cinnamosmolide, however, causes reduced calcium uptake by dorsal root ganglia neurons. The figure shows a representative experiment performed in quadruplicate; five additional experiments yielded similar results.

observations imply that terpenoids may have a more complex action on sensory neurons than does isovelleral. To follow up this observation, isovelleral, cinnamodial, and cinnamosmolide were compared in the cellular assay of $^{45}Ca^{2+}$-uptake. Isovelleral, in accord with previous findings, induced a dose-dependent $^{45}Ca^{2+}$-influx with an EC_{50} value of 105 nM (not shown). The maximal response was comparable to that evoked by capsaicin. By contrast, cinnamodial (Fig. 2) and cinnamosmolide (Fig. 3) yielded complex, at least biphasic $^{45}Ca^{2+}$-uptake response curves.

Cinnamodial evoked a well-measurable $^{45}Ca^{2+}$-accumulation, approaching $47 \pm 9\%$ (mean $\pm$ S.E.M., $n = 6$) of the maximal capsaicin response, at concentrations lower (approximately 30 nM) than that required for half-maximal inhibition of resiniferatoxin binding (600 nM; see Table 1). The initial increase in $^{45}Ca^{2+}$-uptake was prevented by the competitive vanilloid receptor antagonist, capsazepine (10 μM; $76 \pm 8\%$ inhibition; mean $\pm$ range; 2 experiments). Cinnamosmolide induced a somewhat greater level of calcium uptake than did cinnamodial (Fig. 3), reaching $76 \pm 8\%$ (mean $\pm$ S.E.M., $n = 6$) of the capsaicin-evoked level. Capsazepine (10 μM) inhibited the cinnamosmolide-evoked calcium uptake response ($85 \pm 1\%$ inhibition; three experiments).

However, at higher concentrations this initial increase by cinnamodial or cinnamosmolide was superseded by a blockade of the $^{45}Ca^{2+}$-influx response (Figs. 2 and 3; inserts), making the exact determination of EC_{50} values impossible. The approximate EC_{50} values were 30 nM for cinnamodial and 200 nM for cinnamosmolide, respectively (see Figs. 2 and 3, inserts).

3.4. Current-clamp analysis of cinnamodial-evoked responses in cultured rat dorsal root ganglion neurons

In order to determine whether cinnamodial is able to depolarize primary sensory neurons in a capsaicin-like fashion, patch-clamp experiments in the current-clamp mode were performed. Out of the 12 neurons tested, six showed membrane depolarization in response to 10 μM cinnamodial. In four neurons, the depolarization led to generation of action potentials; in two neurons, however, only membrane oscillations were observed. A representative recording obtained from a single neuron is shown in Fig. 4. Panel A displays changes in membrane potential in the neuron during injection (starting at the arrow) of a depolarizing current of 1.0 nA for 500 ms under control conditions. Application of cinnamodial (10 μM) produced an additional depolarization of a few mV, enough to elicit further action potentials during the current application. In every recording, the first action potential is caused by the current itself, whereas the subsequent ones are evoked by cinnamodial. The response to cinnamodial was without delay (panel B); it increased after 8 s (panel C); and then it

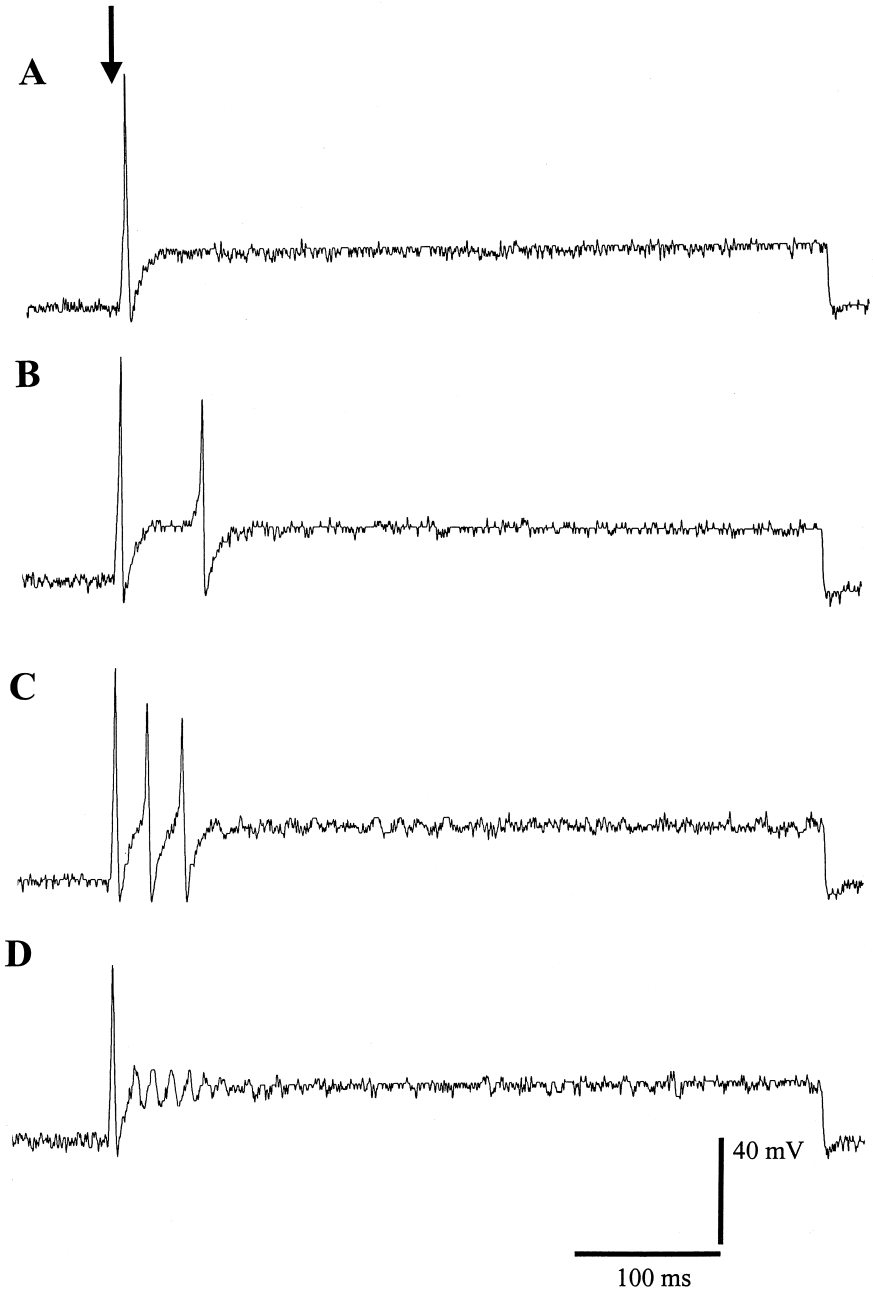

Fig. 4. Cinnamodial-induced membrane depolarizations in an adult rat dorsal root ganglion neuron under current-clamp conditions. The control recording (panel **A**) shows that application of a depolarizing current (1.0 nA for 500 ms, starting at the arrow) depolarized the neuron and evoked a single action potential. When a similar depolarizing current was applied in the presence of 10 μM cinnamodial, an additional depolarization was observed, resulting in action potential generation (panels **B** and **C**) or slow membrane depolarization (recording **D**). The response was either recorded immediately after the onset of cinnamodial administration (**B**), or 8 s (**C**) or 28 s (**D**) later. In every recording, the first action potential is caused by the current administration. The cross-sectional area of the neuron was 595 μm^2; the magnitude of the resting potential was −40 mV.

diminished to slow membrane oscillations after 28 s (panel D). The resting potential of the neurons was 45.0 ± 3.6 mV (mean ± S.E.M.; 12 determinations).

4. Discussion

A receptor for 8-methyl-*N*-vanillyl-6-nonenamide (better known as capsaicin) and related irritant compounds was first postulated based on the fairly strict structure-activity requirements for capsaicin-like activity (Jancsó, 1968; Szolcsányi and Jancsó-Gábor, 1975). The critical role of a vanillyl moiety in capsaicin receptor recognition was later reinforced by the discovery of a naturally occurring ultrapotent capsaicin analog, resiniferatoxin, which is a homovanillyl derivative of resiniferonol 9, 13, 14-orthophenylacetate (Szallasi and Blumberg, 1992). Based on the

shared vanillyl motif essential for bioactivity, the common membrane recognition site for capsaicin and resiniferatoxin congeners was termed the vanilloid receptor (Szallasi and Blumberg, 1996). However, recently a number of pungent terpenoids possessing an unsaturated 1,4-dialdehyde moiety have been found to stimulate capsaicin-sensitive neurons in a vanilloid receptor-mediated fashion (Szallasi et al., 1996), suggesting that the ligand recognition properties of vanilloid receptors are far more complex than thought previously (Bíró et al., 1997; Szallasi and Blumberg, 1996)

Although resiniferatoxin mimics most capsaicin actions, its potency relative to capsaicin ranges from several thousandfold higher (e.g., induction of hypothermia in the rat) to equipotency (e.g., acute pain response, as determined in the rat eye-wiping test) (Szallasi and Blumberg, 1996). Resiniferatoxin also has unique actions, such as the desensitization of pulmonary J1 receptors without prior excitation (Szolcsányi et al., 1990). Although some differences between resiniferatoxin and capsaicin activities may be attributed to dissimilar channel-gating properties (Liu and Simon, 1996), other observations are better explained by postulating the existence of vanilloid receptor heterogeneity (Bíró et al., 1997; Szallasi and Blumberg, 1996). Examples include a variety of kinetically distinct vanilloid-gated conductances in dorsal root and trigeminal ganglion neurons (Liu and Simon, 1996; Petersen et al., 1996), a fraction of which cannot be blocked by the competitive vanilloid receptor antagonist capsazepine, and the differential inhibition by capsazepine and the functional vanilloid antagonist ruthenium red, of vanilloid-induced responses in the isolated rat hindlimb model (Griffiths et al., 1996).

Strong evidence for vanilloid receptor subtypes is the distinct structure-activity relations that capsaicin and resiniferatoxin analogs show for binding to intact sensory neurons and for neuronal calcium uptake (Acs et al., 1996). Resiniferatoxin is approximately 20-fold more potent for binding than for calcium uptake (affinities are 100 pM and 2 nM, respectively), assayed in the same population of cultured neurons, whereas capsaicin induces calcium influx at 30-fold lower concentrations than required for the inhibition of resiniferatoxin binding (Acs et al., 1996). These observations led to the concept of distinct vanilloid receptor subtypes mediating calcium uptake and high-affinity resiniferatoxin binding (Bíró et al., 1997; Szallasi and Blumberg, 1996). This model has recently been reinforced by the discovery of mast-cell lines expressing the vanilloid-sensitive channel but not the high-affinity resiniferatoxin binding site (Bíró et al., 1997).

Recently, a vanilloid receptor (termed VR1) has been cloned (Caterina et al., 1997), which seems to fall in the class of native vanilloid receptors (C-type) mediating calcium uptake. This protein belongs to the family of store-operated calcium channels and is activated by noxious heat (Caterina et al., 1997). Channels related to VR1 may be involved in sensory transduction in *C. elegans* (Colbert et al., 1997), implying a general role for such proteins in chemosensation. Whether the repellency of unsaturated dialdehydes in non-mammals reflects, in part, interaction with targets related to VR1 remains to be determined.

VR1 is not expressed in nodose ganglia (Caterina et al., 1997) a tissue particularly rich in specific resiniferatoxin binding sites (Szallasi et al., 1995). Apparently, C- and R-type vanilloid receptors are not only pharmacologically distinct but also show different tissue distribution. R-type vanilloid receptors are yet to be cloned and the signal transduction pathways that they use remain to be determined. Resiniferatoxin is able to desensitize neurons to subsequent application of resiniferatoxin or capsaicin without causing a detectable calcium current (Acs et al., 1997). This finding may be interpreted as an indication of the R-type receptor being metabotropic. Interestingly, previous studies described an increase in neuronal cGMP following vanilloid administration that seemed to be independent of the calcium fluxes (Wood et al., 1988).

Whereas it is unambiguous based on the present findings that sesquiterpenoid dialdehydes and related bioactive terpenoids can act on both R- and C-type vanilloid receptors, less clear is the receptor subtype selectivity of these compounds. Isovelleral, like capsaicin, is approximately 25-fold more potent in the calcium uptake assay than for inhibition of resiniferatoxin binding, indicating a similar degree of selectivity for C- over R-type receptors. Cinnamodial and cinnamosmolide also seem to be more active in the calcium uptake than in the binding assay, but their affinity is distorted by the inhibition of calcium uptake that overcomes the initial calcium influx. Clearly, more compounds need to be evaluated in both vanilloid receptor assays in order to clarify this question. As yet, the limited quantity of other dialdehyde sesquiterpenes at our disposal has precluded this analysis.

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