Phorboid 20-homovanillates induce apoptosis through a VR1-independent mechanism

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Background: Vanilloids, such as capsaicin and resiniferatoxin (RTX), are recognized at the cell surface by vanilloid receptor type 1(VR1), which has recently been cloned. VR1 mediates the effects of capsaicin and RTX in VR1-expressing cells, but vanilloids can induce apoptosis through a pathway not mediated by VR1. Phorbol 20-homovanillates can be used to investigate cell death induced by vanilloids.

Results: 12,13-Diacylphorbol-20 homovanillates were prepared by the sequential esterification of the natural polyol. Phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPAHV) induced apoptosis in Jurkat cells to the same extent as RTX. Apoptosis was preceded by an increase in intracellular reactive oxygen species and by the loss of mitochondria transmembrane potential. PPAHV-induced apoptosis was mediated by a pathway involving caspase-3 activation and was initiated at the S phase of the cell cycle. The cell-death pathway triggered by VR1 activation was studied in 293T cells transfected with the cloned rat vanilloid receptor. In this system, capsaicin and PPAHV induced cell death by an apparent necrotic mechanism, which was selectively inhibited by the competitive vanilloid receptor antagonist capsazepine. Interestingly, phorbol-12,13-bisnonanoate-20-homovanillate, an analogue of PPAHV, induced cell death in VR1-transfected cells but could not trigger apoptosis in the Jurkat cell line.

Conclusions: Vanilloids can induce cell death through different signalling pathways. The cell death induced in a VR1-independent manner has the hallmark of apoptosis, whereas the cell death mediated by vanilloids binding to VR1 is seemingly necrotic. Phorboid homovanillates that have antitumour and anti-inflammatory activities but lack the undesirable side effects of the natural vanilloids could be developed as potential drugs.

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Introduction

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide, CPS), which is responsible for the pungency of hot pepper, and resiniferatoxin (RTX), a complex diterpenoid isolated from certain succulent African *Euphorbias*, are considered the archetypal vanilloids. These compounds interact at specific membrane recognition sites (vanilloid receptors) expressed almost exclusively by primary sensory neurons involved in nociception and neurogenic inflammation [1], and in tracheobronchial tissues [2,3].

It is generally accepted that specific vanilloid receptors mediate the effects of CPS and RTX in some cell systems. Thus, transient exposure of nociceptor terminals to capsaicin leads to excitation and desensitisation in dorsal root ganglion neurons and to an increase in intracellular calcium [4–6]. These biological activities underlie the use of capsaicin as an analgesic agent in the treatment of painful disorders such as peripheral neuropathies and rheumatoid arthritis [7–9]. The recent cloning of a vanilloid receptor, termed VR1 (vanilloid receptor subtype 1), has shown that this receptor belongs to the family of putative store/operate calcium channels and it is expressed exclusively in trigeminal dorsal root sensory ganglia [6]. VR1 also is a receptor for the endogenous cannabinoid anandamide [10].

In addition to having these biological activities, CPS is an inhibitory quinone analogue that can inhibit NADH oxidase found in plasma membranes [11], and can induce apoptosis by a vanilloid-receptor-independent pathway in transformed cells and in activated T cells [12–14]. We have shown recently that CPS-induced apoptosis in transformed cells was preceded by an increase in reactive oxygen species (ROS) and subsequent disruption of the mitochondrial transmembrane potential ($\Delta\Psi_m$), a process that can be related to mitochondrial permeability transition pore opening (PT), as PT inhibitors (such as Bcl-2

and cyclosporine A) inhibit this disruption [13,14]. Furthermore, CPS has also been shown to have immunomodulatory effects, as indicated by its ability to modulate lymphocyte proliferation and immunoglobulin production [15–17], and to induce interleukin-4 (IL-4) production by mast cells [18]. Taken together, these results indicate that CPS, and perhaps other vanilloids, have pleiotropic activities, presumably mediated by the activation of cellular targets other than VR1, which can be expressed not only in sensory neurons but also in other cell types [11].

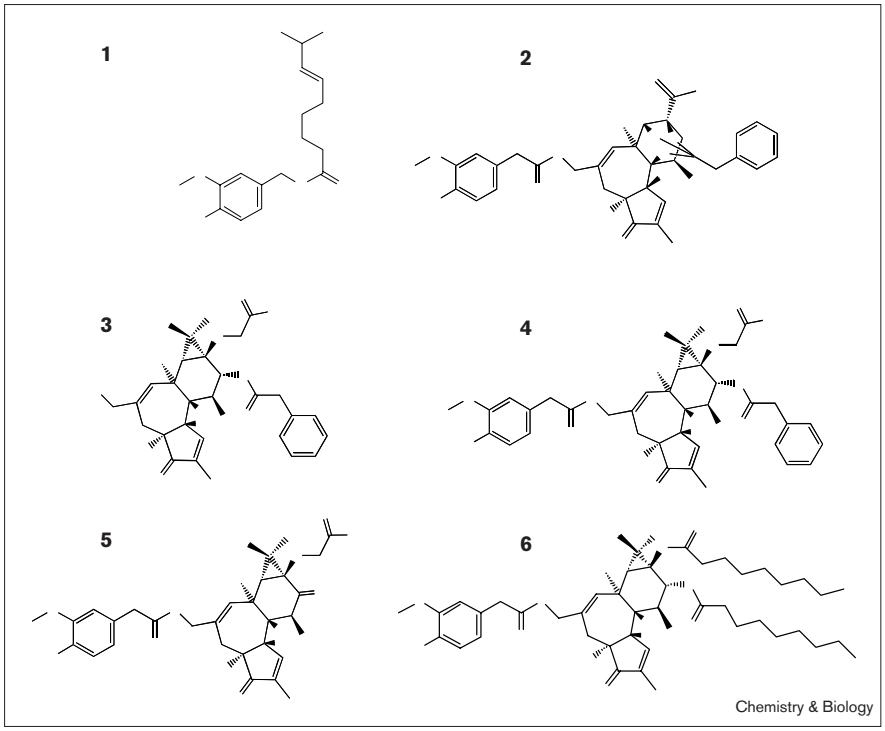
In the past decade, the information obtained on the existence of different subtypes of receptors came mainly from two sources: binding experiments using [^{3}H]RTX, an ultrapotent CPS analogue, and capsazepine, a competitive CPS antagonist [9]; and through the use of CPS analogues such as phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPHAV), which binds to the CPS receptor in a non-cooperative manner on sensory neurons. Unlike CPS, however, in different biological assays, it is not pungent topically and does not result in hypothermia when injected in animal models [19,20]. Szallasi *et al.* [21,22] have shown that cloned VR1 mediates both the R-type binding and the C-type calcium response in dorsal root ganglion neurons. Moreover, it has been shown that phorboid-20 homovanillates are useful for evaluating structure–activity relations at the vanilloid receptor in both rat trigeminal ganglion neuron [23] and in other types of cells transfected with the gene encoding VR1 [24]. Surprisingly, the effects of these phorbol–RTX hybrids on non-neuronal cells have not been investigated to date.

Here, we show that the vanillyl moiety of CPS, RTX and PPAHV is required to produce intracellular ROS generation, to open the mitochondria megachannels (called permeability transition, PT), and to induce apoptosis in human transformed cell lines that do not express VR1. We also show that PPAHV-induced apoptosis is mediated via a pathway that involves caspase-3 and occurs at the specific cell-cycle checkpoint that controls G_1–S phase transition. The cell death mediated through VR1 was studied in 293T cells transfected with the cloned rat vanilloid receptor. This form of cell death induced by both CPS and PPAHV could be inhibited by the competitive vanilloid receptor antagonist capsazepine. In contrast to RTX and CPS, PPAHV also activates the transcription factor AP-1, at least in part via the mutogen-activated protein kinase signalling pathway. Nevertheless, PPAHV-mediated apoptosis does not require AP-1 activation and is not affected by MAPK inhibition. Possible mechanisms by which natural and phorbol-derived vanilloids can induce apoptosis or necrosis are also discussed.

Results

The capacity of different vanilloid analogues to induce apoptosis in human transformed cell lines has been investigated. The compounds used in this study are shown in Figure 1: **1**, CPS; **2**, RTX; **3**, phorbol 12-phenylacetate

Figure 1



Chemical structures of **1**, CPS, **2**, RTX, **3**, PPA, **4**, PPAHV, **5**, DPAHV and **6**, PBNHV.

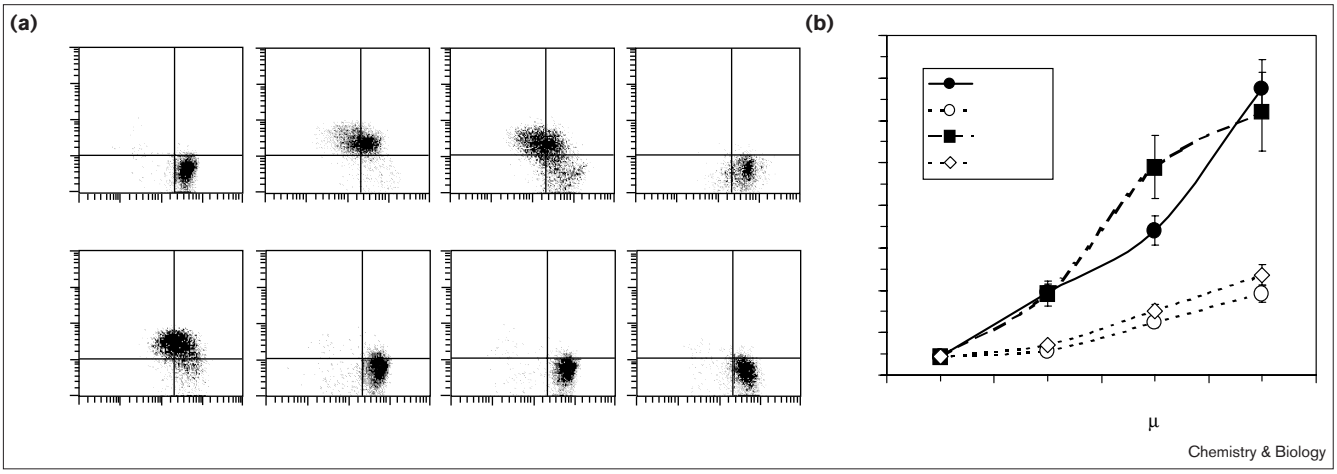
13-acetate (PPA); **4**, PPAHV; **5**, 12-dehydrophorbol 13-acetate 20-homovanillate (DPAHV); and **6**, phorbol-12, 13-bisnonanoate-20-homovanillate (PBNHV).

Induction of apoptosis by capsaicin and vanilloids analogues in Jurkat cells

It is now accepted that a breakdown in $\Delta\Psi_m$ and the generation of ROS are invariant features of early apoptosis [25–30]. Thus, we studied the role of $\Delta\Psi_m$ and ROS generation in the apoptotic pathway induced in Jurkat T cells by the following compounds: CPS (200 μM), RTX (10 μM); ROPA (aresinferol *o*-phenylacetate, a 20-decylated derivative of RTX) (10 μM), DPAHV (10 μM), PPAHV (10 μM), PBNHV (10 μM) and PPA (10 μM) in the presence of vanillate (10 μM). The cells were treated for 6 h, and ROS generation and $\Delta\Psi_m$ dissipation were detected by double staining experiments, using hydroethidine (HE; nonfluorescent), which becomes ethidium (Eth; red fluorescent) after its oxidation via ROS, and $DiOC_6(3)$ (green fluorescent), a cationic probe that accumulates in mitochondria as a function of its potential [31]. After treatment for 18 h, hypodiploidy (i.e., loss of fragmented DNA) was analysed, using propidium iodide (PI) staining, as a marker for apoptosis.

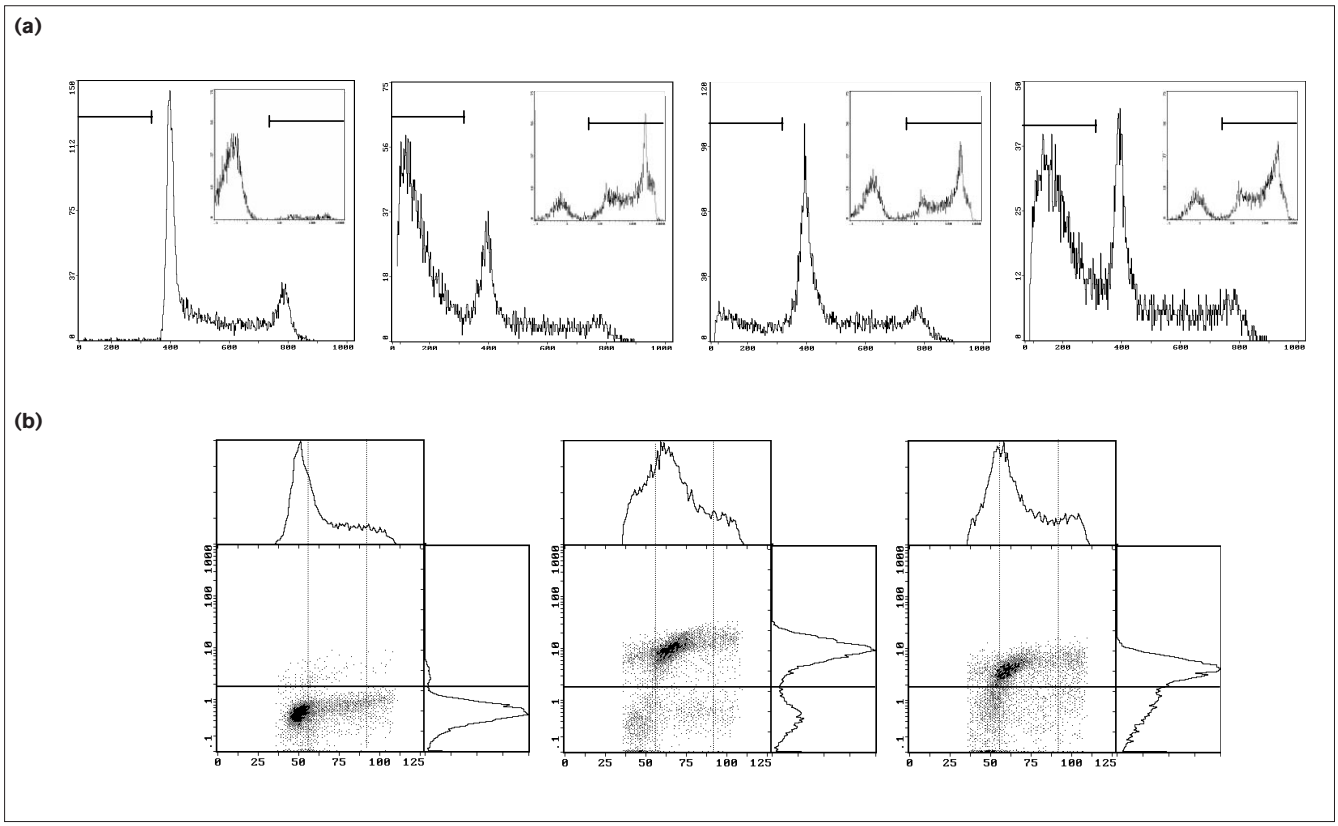
In these experiments, untreated cells were taken as controls, with a high $\Delta\Psi_m$ ($DiOC_6(3)^{high}$) and low levels of intracellular ROS $(HE\rightarrow Eth)^{low}$ (Figure 2). As expected, CPS and RTX induced an increase in the percentages of $DiOC_6(3)^{low}/(HE\rightarrow Eth)^{high}$ and $DiOC_6(3)^{high}/(HE\rightarrow Eth)^{high}$ cells, whereas the percentage of $DiOC_6(3)^{low}/(HE\rightarrow Eth)^{low}$ cells did not change, when compared with untreated cells. The role of the vanillyl moiety of these hybrid molecules in this apoptotic pathway was demonstrated by the finding that PPAHV was as efficient as RTX at inducing first ROS generation, and then $\Delta\Psi_m$ disruption and apoptosis in Jurkat cells, whereas neither PPA in the presence of vanillate nor DPAHV affected these parameters. DPAHV is a hybrid molecule with a nonactive phorbol moiety [19]. Moreover, ROPA was ineffective at inducing ROS generation and apoptosis in this cell line. Together, these results show that the vanillyl moiety of these compounds is responsible for the biological effects measured in our model, but the other moiety of the molecule (alkyl in CPS or diteperne in RTX and PPAHV) is also required (Figure 2a). Apoptosis was also measured using the TUNEL method (see the Materials and methods section for details), and we found that both RTX and PPAHV induced apoptosis in Jurkat cells in a dose-dependent manner, with similar relative potencies. Interestingly, PBNHV, which contains an active phorbol moiety, did not induce a significant level of apoptosis in the Jurkat cell line (Figure 2a,b). The low levels of apoptosis found with the higher doses of ROPA and PBNHV is not mediated by an increase in intracellular ROS and it is not statistically significant.

Figure 2



Induction of apoptosis by vanilloids in Jurkat cells. **(a)** Jurkat cells were treated with CPS (200 μM), RTX (10 μM), ROPA (10 μM), PPAHV (10 μM), PBNHV (10 μM), PPA (10 μM) plus vanillate (10 μM), and DPAHV (10 μM). After a 6 h treatment, half of the cells were collected and the simultaneous $\Delta\Psi_m$ disruption and ROS generation detected using cytofluorimetry. The results represent the percentage of cells obtained in biparametric histograms delimited by four compartments: $\Delta\Psi_m{}^{high}$ (normal cells, bottom right compartment); $\Delta\Psi_m{}^{low}$ (bottom left); $(HE\rightarrow Eth)^{high}$ (ROS-generating cells, top right), and $(HE\rightarrow Eth)^{high}/\Delta\Psi_m{}^{low}$ (pre-apoptotic cells, top left compartment). Apoptosis was measured after 18 h of treatment by cell-cycle analysis with PI staining (percentage of apoptotic cells between brackets). Results are representative of five independent experiments. **(b)** Detection of DNA strand breaks using the TUNEL method. Jurkat cells were treated with increasing concentrations of the indicated compounds for 18 h and the percentage of apoptotic cells determined. Values are means ± SD of three independent experiments.

Figure 3



(a) The caspase-3 inhibitor Ac-DEVD-cmk inhibits PPAHV-induced apoptosis. Jurkat cells were treated with PPHAV (20 μM) in the presence or absence of the caspase inhibitors acetyl-DEVD-cmk (100 μM) and Z-YVAD-cmk (100 μM) for 18 h, and the percentage of subdiploid cells was detected using flow cytometry. Cell death was also determined by PI staining as described in the Materials and methods section. **(b)** S-phase dependency for apoptosis induced by PPAHV and RTX. Jurkat cells were stimulated with PPAHV and RTX (both at 20 μM) for 18 h and the cell cycle and the DNA strand breaks analysed using flow cytometry. Results are representative of three separate experiments.

Apoptosis induced by PPAHV in Jurkat cells is mediated by caspase-3 activation and occurs at the S phase of the cell cycle

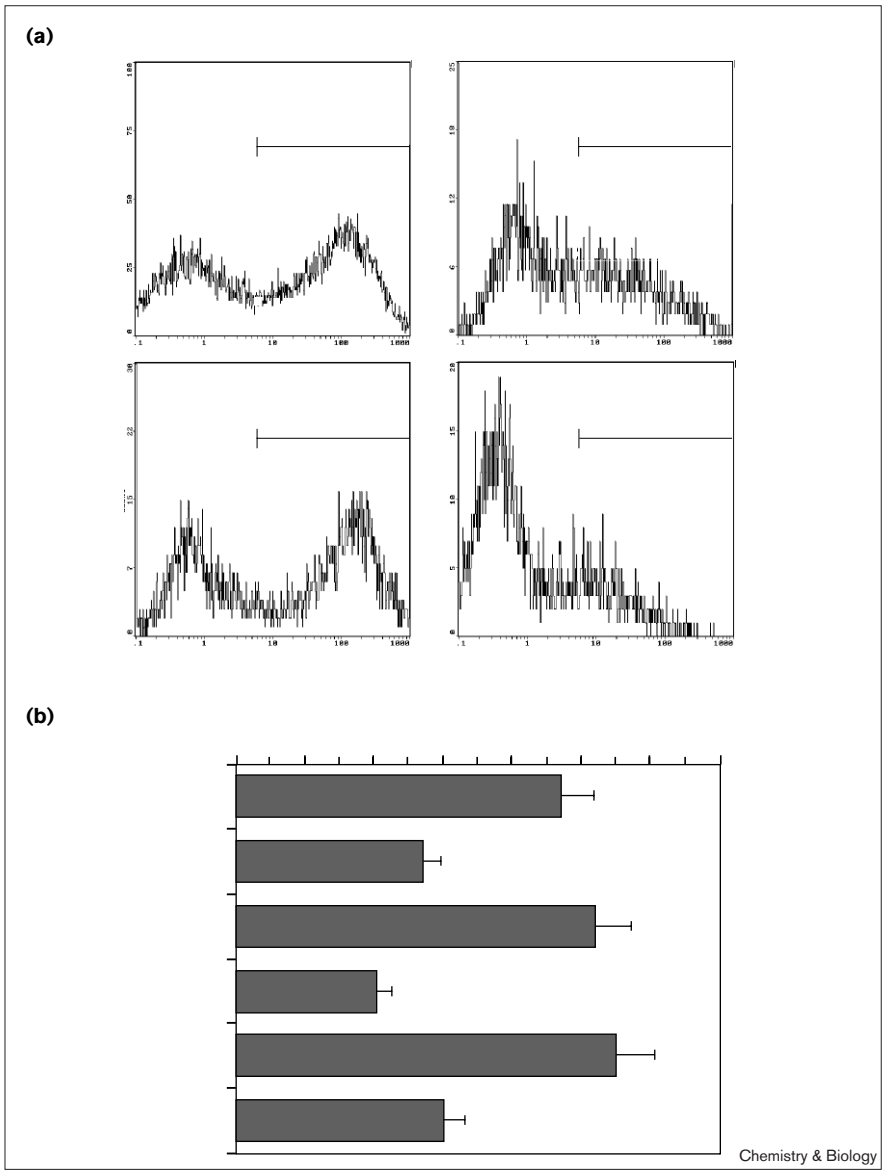
The caspase family comprises post-aspartate-cleaving cysteine proteases that have been shown to be required for apoptosis in a number of experimental systems [32–34]. To establish the involvement of caspases in PPAHV-mediated apoptosis of Jurkat cells, we pre-incubated cells with the tetrapeptide caspase inhibitors acetyl-DEVD-cmk (cmk, chloromethylketone) or Z-YVAD-cmk before PPHAV treatment. Z-YVAD-cmk, which was reported to specifically inhibit caspase-1 and related enzymes, had little effect on the increase in the percentage of subdiploid cells induced by PPAHV (at 20 μM). In contrast, the same doses of acetyl-DEVD-cmk, which is more specific for the inhibition of caspase-3, a member of the CED-3 subfamily of caspases, abolished the apoptosis induced by PPAHV. Nevertheless, PPAHV-treated cells died even when caspase-dependent DNA loss was blocked (Figure 3a). This caspase-independent death has been termed necrosis, and it is also triggered after the loss of $\Delta\Psi_m$ [30,33,35], suggesting that intracellular ROS induced by PPHAV and other vanilloids can be a signal that regulates both necrotic and apoptotic cell death.

The involvement of cell cycle control in apoptosis induced by PPAHV and RTX in Jurkat cells was studied by double staining with PI and FITC–dUTP, as described in the Materials and methods section. This method allowed us to determine in which phase of the cell cycle DNA fragmentation occurs. Thus, in Figure 3b we show that most of the DNA fragmentation was detected in S phase in Jurkat cells treated for 18 h with either PPAHV or RTX (both at 20 μM). We also detected, however, a small but significant percentage of cells that undergo DNA fragmentation at G_o/G_1 phase when treated with both homovanillates.

Cell death induced by phorboid 20-homovanillates in VR1-transfected 293T cells

It has been shown that CPS induces cell death in 293T cells transfected with rat VR1. This cell death occurs

Figure 4

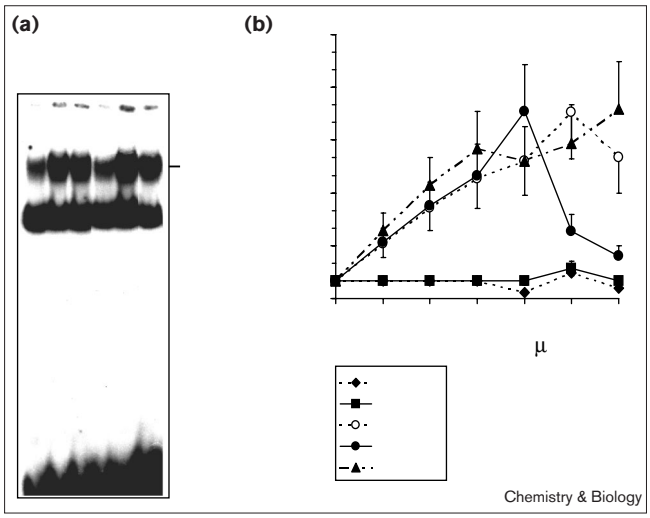


Capzasepine inhibits PPAHV and capsaicin-induced cell death in VR1-transfected cells. **(a)** Capsaicin induced cell death in VR1-transfected 293T cells. The cells were co-transfected with the plasmid pEGFP-C1 and either pcDNA-3 or pcDNA3-VR1 plasmids. After 48 h, the cells were stimulated with CPS (3 μM) for 6 h and the adherent live cells analysed for the expression of the green fluorescent protein by cytometry. Results are representative of five seperate experiments. **(b)** 293T cells were co-transfected with the pEGFP-C1 and pcDNA-VR1 plasmids and 48 h after transfection the cells were stimulated for 6 h with the indicated compounds. The percentage of green fluorescent cells was analyzed as described above. Values are means ± SD of three independent experiments.

rapidly and with a concentration of CPS that does not induce apoptosis in the absence of VR1 [6,13]. To evaluate the role of VR1 in the cell death induced by synthetic vanilloids in 293T cells, we co-transfected the cells either with an expression plasmid containing the entire VR1 cDNA or with the empty vector (pcDNA3), and with an equimolar concentration of the plasmid pEGFP-C1. The cells were stimulated, 48 h after transfection, with CPS (3 μM) for 6 h, and the percentage of green fluorescent cells was detected using flow cytometry. We found that this cell type is transiently transfected with high efficiency; Figure 4a illustrates that more than 50% of the cells express the green fluorescent protein. The percentage of positive cells did not change with CPS treatment in the pcDNA3 co-transfected cells, whereas a clear reduction in this percentage was observed in cells co-transfected with the plasmid pcDNA3-VR1.

We also found that PPAHV (1 μM) was as effective as CPS in the induction of cell death in VR1-transfected 293T cells. The cell death induced by both vanilloids was completely inhibited in the presence of capsazepine (10 μM), a competitive CPS antagonist (Figure 4b). Interestingly, the phorbol homovanillate PBNHV, which binds VR1 and has an active phorbol moiety [24], induces cell death in the VR1-transfected 293T cells at 1 μM (Figure 4b), but did not induce ROS generation or $\Delta\Psi_m$ disruption at doses up to 10 μM in either Jurkat or 293T

Figure 5



Induction of AP-1 activation by phorboid 20-homovanillates.
(a) Nuclear extracts were prepared from Jurkat cells treated with PMA (50 ng/ml), PPAHV (10 μM), RTX (10 μM), PPA (10 μM) and PBNHV (10 μM) for 3 h, and then analyzed for AP-1 binding by EMSA.
(b) HeLa cells were transiently transfected with an AP-1-dependent luciferase reporter construct and 24 h later stimulated with different concentrations of the agonists indicated. After 24 h stimulation, the cells were lysed and the luciferase activity measured. The transcriptional activity was expressed as x-fold transactivation over the values obtained with non stimulated cells. Values are means ± SD of three independent experiments.

cells (Figure 2a and data not shown). Altogether, these results indicate that vanilloids may induce cell death by at least two mechanisms: the first is mainly mediated by apoptosis and occurs in transformed cells that do not express the vanilloids receptor type I; the second, which requires lower doses of vanilloids, is effective in VR1-expressing cells and is likely to be mediated by a rapid necrotic pathway.

Phorboid 20-homovanillates induce AP-1 activation in transformed cells

The activity of the transcription factor AP-1 is regulated by *de novo* synthesis of Jun and Fos, as well as by post-transcriptional modification of c-Jun through phosphorylation of its transactivation domain [36–38].

Members of the MAPK family [38,39] mediate the regulation of AP-1 in response to external stimuli. The best characterized MAPKs are the extracellular signal-regulated protein kinases 1 and 2 (ERK 1 and 2) and the c-Jun amino-terminal kinases (JNK; also known as stress-activated protein kinase, SAPK). As ERK1 and 2 and JNK can be activated by phorbol esters [40–43], we studied the possible activation of the AP-1 transcription factor in Jurkat and in HeLa cells stimulated with different phorboid 20-homovanillate hybrids molecules.

Nuclear extracts from phorbol myristate acetate (PMA)-treated Jurkat cells exhibited a strong binding to an end-labelled AP-1 probe (Figure 5a). Anti-Jun and anti-Fos antiserum were used in cold competition assays leading to a supershift, further demonstrating the specificity of this binding (data not shown). A similar increase in AP-1–DNA binding was detected in Jurkat cells after 3 h of treatment with PPAHV (10 μM), PBNHV (10 μM) and PPA (10 μM), demonstrating that coupling of the homovanillate group to PPA did not interfere with its ability to activate AP-1. Moreover, we did not detect a clear increase in the binding of AP-1 to DNA in the nuclear extracts from RTX-stimulated cells (Figure 5a).

In order to correlate these results with transcriptional activities, HeLa cells were transiently transfected with a luciferase reporter construct under the control of three AP-1 binding sites. Cells were treated, 24 h after transfection, for a further 24 h with increasing concentrations of RTX, DPAHV, PPA, PPAHV and PBNHV separately, and the luciferase activity was measured. Both PPA and PBNHV, which, at the doses tested, induce neither ROS generation nor apoptosis, increased AP-1-dependent transcription up to tenfold induction over the basal levels (Figure 5b). By contrast, neither RTX nor DPAHV (which is inactive as a phorbol ester) was able to activate gene transactivation driven by AP-1. It is interesting to note that PPAHV activated AP-1 transcription in a dose-dependent manner, peaked at 10 μM, and then decreased with doses of 20 and 40 μM, when ROS generation and apoptosis are already detected (Figure 2a,b).

ROS generation and disruption of $\Delta\Psi_m$ induced by PPAHV is independent of AP-1 activation and is not protected by ERK1/2 activation

The role of AP-1 activation in the apoptotic pathways activated by different stimuli is not yet clear [13,44–47]. Moreover, activation of protein kinase C (PKC) can induce, via the c-Raf pathway, the activation of the MAPK ERK1/2 [48], which, in some models, prevents apoptosis and promotes cell survival [49–51]. As we found that PPHAV induced AP-1 activation and apoptosis, we studied whether or not AP-1 had a role in the apoptotic pathway induced by this phorboid homovanillate. To address this question, we preincubated Jurkat cells with the ERK inhibitor PD98059 (50 μM). The cells were then stimulated with PPHAV (20 μM) for 2 h to detect AP-1 binding and for 6 h to study ROS generation and $\Delta\Psi_m$ disruption. Figure 6a illustrates that both PMA and PPAHV induced AP-1 binding to DNA, and that the ERK inhibitor prevented the binding of AP-1 to DNA almost completely in cells activated by PPAHV.

On the contrary, the same doses of the inhibitor did not prevent the biochemical mechanisms leading to apoptosis in PPAHV-treated cells, that is PD98059 alone did

not induce any changes in the levels of intracellular ROS and did not affect the mitochondria transmembrane potential (Figure 6b).

Discussion

Natural products can provide important leads to develop new drugs and to discover the specific cellular targets underlying their activities. Pungent vanilloids have been shown to act through VR1 [6], but there is mounting evidence that their effects are not exclusively mediated by this temperature-sensitive cation channel. To further explore this issue, natural or synthetic vanilloids lacking pungent activity and/or VR1-binding capacity and screening assays that can differentiate between VR1-dependent and VR1-independent biological actions are needed.

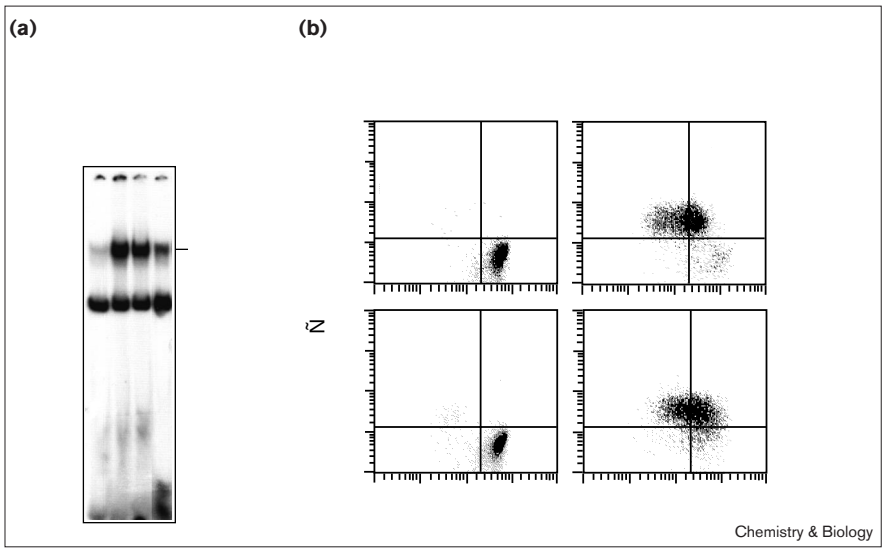
Here, we show that cell-death pathways induced by vanilloids are biochemically different, depending on the expression or lack of VR1. We have developed two different, rapid screening systems that allowed us to conclude that vanilloid-induced cell death in non-VR1-expressing cells has the hallmark of apoptosis, whereas a necrotic pathway mediates cell death in VR1-transfected cells.

We have found that different but structurally related vanilloids (natural and synthetic) can induce apoptosis in tumour cells by a VR1-independent pathway. The molecular basis of this activity seems to be a specific interaction of vanilloids with the plasma membrane redox system (PMOR), which is upregulated in transformed and in activated peripheral T cells [11,14]. This interaction may result in the inhibition of NADH oxidase enzymatic activity, for which oxygen is the terminal electron acceptor. Our results are consistent with the concept that CPS and RTX interfere with the CoQ-binding site of the NADH oxidase system, leading to the redirection of normal electron flow in the complex, thereby generating an excess of ROS that eventually activates the apoptotic pathway [11–14,52,53]. In contrast, in VR1-transfected cells the cell death induced by vanilloids occurs rapidly and is not preceded by an increase in ROS generation before the disruption of $\Delta\Psi_m$ (data not shown). Moreover, the concentrations of vanilloids required for killing VR1-expressing cells are at least tenfold lower for phorboid homovanillates and 100-fold lower for CPS than the doses required for induction of apoptosis in transformed cells that do not express this receptor.

In the apoptotic pathway induced independently of VR1 expression, the vanillyl moiety of natural and synthetic vanilloids is responsible for the induction of cell death; the proper delivery of the vanillyl moiety to cells by the rest of the molecule is, however, also important. Accordingly, Jung *et al.* [54] have shown recently that CPS binds to the intracellular domain of VR1. These results do not exclude the possibility that other vanilloids can interact with the extracellular domain of this receptor. Nevertheless, they demonstrate that vanilloids can be taken up by cells and exert their function on the inner side of the plasma membrane.

The results obtained with phorboid 20-homovanillates demonstrate that the phorbol template can magnify vanilloid activity. Apoptosis induced by PPAHV is, however, independent of AP-1 activation, because PPA, a compound that does not induce ROS, was as potent as PPAHV in activating AP-1. Moreover, the ERK inhibitor PD98059 inhibited AP-1 binding to DNA, but did not inhibit ROS generation or the mitochondrial function in PPAHV-treated Jurkat cells. In addition, DPAHV, another phorboid-20-homovanillate that is not active as a phorbol ester,

Figure 6



Induction of ROS by PPAHV in Jurkat cells is independent of AP-1 DNA binding activity. **(a)** PD98059 (50 μM) inhibits the AP-1 binding in the nuclear extracts of Jurkat cells stimulated for 3 h with PPAHV. (**b)** PD98059 (50 μM) does not affect either ROS generation or $\Delta\Psi_m$ dissipation in PPAHV-treated cells for 6 h.

did not activate AP-1; neither did it induce ROS nor apoptosis. This is consistent with the fact that PKC is a cytosolic enzyme that, upon activation, is translocated to the inner side of the cell surface, where it can phosphorylate multiple substrates [55,56]. In this context, the vanilloid moiety of the molecule could interact with the PMOR system, inhibiting the NADH oxidase activity and generating ROS.

The binding sites for the alkyl moiety of CPS and the diterpene core of RTX are not well characterized. Furthermore, it has recently been shown that RTX, but not CPS, binds a kinase distinct from the PKC isotypes α, β, γ, δ and ε [57]. This result is in agreement with our finding that RTX did not activate AP-1. In phorbol–RTX hybrids, the esterification pattern of the cyclohexane ring is important for the induction of ROS and apoptosis in transformed cells. Thus, the phorboid homovanillate PBNHV, a compound which is able to activate AP-1 binding to DNA and AP-1-dependent transactivation but which is unable to induce ROS generation, could nevertheless induce cell death in VR1-expressing cells. This finding implies differences between the effects of vanilloids on VR1 and the inhibition of the PMOR system. A possible explanation is that the large lipophilic moiety of PBNHV renders the PMOR system inaccessible to the vanilloid moiety.

Capsaicin has been used topically as an analgesic for a long time, but the systemic use of vanilloids is limited by their neurotoxicity and their capacity to induce neurogenic inflammation [1]. Upon activation, CPS-sensitive nerves transmit signals to the central nervous system and release pro-inflammatory neuropeptides such as substance P and calcitonin-gene-related peptide into the periphery [58,59]. These neuropeptides can induce mast cells degranulation with the consequent release of proteoglycans, histamine and serotonine, and cytokines such as interleukins and tumour necrosis factor-α [60,61]. Thus, the identification of natural or synthetic vanilloids endowed with apoptotic activities but unable to bind and activate the VR1 may be of special interest to design new antitumour and anti-inflammatory drugs.

Significance

Vanilloids are a class of structurally diverse compounds exemplified by capsaicin (CPS) and resiniferatoxin (RTX). In the past two decades, these compounds have been extensively investigated for their analgesic potential in the treatment of painful disorders, a biological effect that is seemingly mediated by the activation of the vanilloid receptor type I (VR1), a heat-sensitive ion channel expressed mainly in nociceptive neurons. We and others have shown that CPS and RTX can induce apoptosis in transformed cells that do not express VR1. In this report, we demonstrate that vanilloid-induced apoptosis occurs mainly at the S phase of the cell cycle and is mediated by a caspase-3-dependent mechanism. In contrast, cell death induced by vanilloids through VR1 activation is mediated by a necrotic mechanism that can be clearly differentiated from the apoptotic pathway. Employing complementary methods to screen the apoptotic properties of vanilloids, we discovered that, despite having a much reduced affinity for VR1, certain phorboid homovanillates can induce apoptosis with the same potency as the ultrapotent vanilloid RTX. These compounds therefore represent interesting leads for the development of new anticancer drugs.

Materials and methods

Cell lines and reagents

Jurkat cells (ATCC, Rockville, MD, USA) were maintained in exponential growth in RPMI-1640 medium (Bio-Whittaker, VerViers, Belgium), and the human embryonic kidney-derived 293T cells and the cervix cancer HeLa cells (ATCC) in DMEM (Bio-Whittaker). The culture media were supplemented with 10% heat-inactivated foetal calf serum, 2 mM L-glutamine and the antibiotics penicillin and streptomycin (Gibco, Paisley, Scotland). [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from ICN (Costa Mesa, CA, USA). Capsazepine and resiniferonol (ROPA) was purchased from Alexis Co (Läufelfingen, Switzerland). The cell-permeable inhibitors of caspase-1 (Z-YVAD-cmk), and caspase-3 (acetyl-DEVD-cmk) were obtained from Bachem (Switzerland). FITC-12-deoxy-2-uridine triphosphate (FITC-dUTP) and terminal deoxynucleotidetransferase (TdT) were obtained from Boehringer Mannheim (Germany). The ERK inhibitor PD98059, was from Calbiochem (La Jolla, CA, USA). All other reagents not cited above or later were obtained from Sigma Chemical Co. (Barcelona, Spain).

Synthesis of phorboid 20-homovanillates

Synthesis of PBNHV as representative: phorbol was reacted with a large excess (12 mol. equiv.) of nonanoyl chloride (pyridine, room temperature [RT], 24 h, 75%). The 12,13,20-triester was then transesterified with 0.001 N $HClO_4$ in methanol (RT, 24 h, 89%), and the resulting 12,13-diester was esterified with MEM2-Methoxyethoxymethyl-homovanillic acid (DCC, dicyclohexylcarbodiimide DMAP 4-dimethylaminopyridine, and CH_2Cl_2, 90%). The MEM group was finally removed with $SnCl_4$ in THF [tetrahydrofuran] (4 mol. equiv, RT, 4 h, 71%), PPA (phorbol 12-phenylacetic 13-acetate), DPAHV (12-dehydrophorbol 13-acetate 20- homovanillate) and PPAHV (Phorbol 12-phenylacetate 13-acetate 20-homovanillate) were synthesized as previously reported [19].

Determination of mitochondrial transmembrane potential and ROS generation

To study the mitochondrial transmembrane potential and ROS generation, we incubated the cells (10^6/ml) in phosphate-buffered saline (PBS) with $DiOC_6(3)$ (green fluorescence) (20 nM) (Molecular Probes Europe) and dihydroethidine (HE) (red fluorescent after oxidation) (2 μM) (Sigma) for 20 min at 37°C, followed by analysis on an Epics XL Analyzer (Coulter, Hialeah, FL, USA). Where indicated, the percentage of live cells and those that had undergone death was determined by incubating an aliquot of the cells with 10 μg/ml of PI for 2 min at RT. Cells permeable to PI (i.e., dead) appear as red fluorescence by flow cytometry.

Determination of nuclear DNA loss and cell cycle analysis

The percentage of cells undergoing chromatinolysis (subdiploid cells) was determined by ethanol fixation (70%, for 24 h at 4°C). Then, the cells were washed twice with PBS containing 4% glucose and subjected to RNA digestion (using RNAse-A, 50 U/ml) and PI (20 μg/ml) staining in PBS for 1 h at RT. The cell cycle was analyzed by cytofluorimetry as previously described [62]. With this method, low molecular weight DNA leaks from the ethanol-fixed apoptotic cells and the subsequent staining allows determination of the percentage of subdiploid cells (the sub-G_0/G_1 fraction).

Detection of DNA strand breaks using the TUNEL method

The percentage of apoptotic cells was also measured using the TUNEL method, as previously described [63], with minor modifications. Briefly, cells (1×10^6) were fixed in 4% paraformaldehyde in PBS for 24 h at 4°C, washed twice in PBS and permeabilized in 0.1% sodium citrate containing 0.1% Triton X-100 for 2 min Fixed cells were washed three times in PBS and resuspended in a final volume of 50 µl of TUNEL buffer (0.3 nmol FITC-dUTP, 3 nmol dATP, 50 nmol $CoCl_2$, 5 U TdT, 200 mM potassium cacodylate, 250 µg/ml bovine serum albumen (BSA) and 25 mM Tris-HCl pH 6.6). The cells were incubated for 1 h at 37°C and then washed twice in PBS and analyzed by flow cytometry. To determine both DNA strand breaks and the phase of the cell cycle cells were in, TUNEL-stained cells were counterstained with PI and treated with RNAse, as described above, prior to cytofluorimetric analysis. In this method, fixation in formaldehyde prevents extraction of low molecular weight DNA from apoptotic cells and thus the cell cycle distribution estimates both apoptotic and nonapoptotic cells [63].

Isolation of nuclear extracts and mobility shift assays

Jurkat cells were cultured at 2×10^6/ml and stimulated with the agonists in complete medium, as indicated. Cells were then washed twice with cold PBS and proteins from nuclear extracts isolated as previously described [13]. Protein concentration was determined by the Bradford method (Bio-Rad, Richmond, CA, USA). For the electrophoretic mobility shift assay (EMSA), a double-stranded oligonucleotide containing the AP-1 site of the metallothioneine promoter was used. The binding reaction mixture contained 3 µg of nuclear extract, 0.5 µg poly(dI-dC) (Pharmacia Fine Chemical, Piscataway, NJ, USA), 20 mM HEPES pH 7, 70 mM NaCl, 2 mM DTT, 0.01% NP-40, 100 µg/ml BSA, 4% Ficoll, and 100 000 cpm of end-labelled DNA fragments in a total volume of 20 µl. After a 20 min incubation at 4°C, the mixture was electrophoresed through a native 6% polyacrylamide gel containing 89 mM Tris-borate, 89 mM boric acid and 1 mM EDTA. Gels were pre-electrophoresed for 30 min at 225 V and then for 2 h after loading the samples. These gels were dried and exposed to XAR film at −70°C.

Transient transfections and luciferase activity

293T cells (10^5/ml) were transiently co-transfected with the pEGFP-C1 plasmid encoding a brighter fluorescent variant of the Green Fluorescence Protein (Clontech, Palo Alto, CA) and either the plasmid encoding the complete rat cDNA VR1 [6] or the empty vector, pcDNA3 (Invitrogen, Leek, The Netherlands). The transfections were performed using lipofectamine reagent (Life Technologies, Madrid, Spain), according to the manufacturer's recommendations. Forty eight hours after transfection, the cells were stimulated with the different compounds for 6 h and the percentage of adherent green fluorescent cells (live cells) measured by flow cytometry. To determine AP-1-dependent transcription, HeLa cells were transfected with the AP-1-luciferase (0.5 µg/ml) reporter plasmid that was constructed by inserting three copies of a SV40 AP-1-binding site into the Xho site of pGL-2 promoter vector (Promega Co., WI, USA). Twenty four hours post-transfection, the cells were stimulated with the indicated agonists for 24 h and then lysed in 25 mM Tris-phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM dithiothreilol, 1% Triton X-100, and 15% glycerol. Luciferase activity was measured in a luminometer (Lumat Berthold, Bad Wildbad, Germany) following the instructions of the luciferase assay kit (Promega Co.). The background obtained with the lysis buffer was subtracted in each experimental value. All the experiments were repeated at least three times.

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