



Functional and desensitizing effects of the novel synthetic vanilloid-like agent 12-phenylacetate 13-acetate 20-homovanillate (PPAHV) in the perfused rat hindlimb

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1 In the present study, the effects of the novel vanilloid agonist, 12-phenylacetate 13-acetate 20-homovanillate (PPAHV), on oxygen consumption (VO_2) and vascular resistance (perfusion pressure, PP) were investigated in the constant flow, perfused rat hindlimb. The acute desensitizing properties of this novel synthetic agent were also examined.

2 Maximum stimulation of VO_2 was produced by $0.2 \mu M$ PPAHV (ΔVO_2 , $0.83 \pm 0.06 \mu mol g^{-1} h^{-1}$) and was accompanied by mild vasoconstriction (increase in PP; 8.0 ± 1.1 mmHg). The highest concentration of PPAHV tested ($2 \mu M$) caused inhibition of VO_2 (ΔVO_2 , $-2.73 \pm 0.51 \mu mol g^{-1} h^{-1}$) and strong vasoconstriction (ΔPP , 42.0 ± 1.2 mmHg).

3 Capsazepine ($10 \mu M$) caused a parallel shift to the right of both VO_2 and PP concentration-response curves for PPAHV ($pK_b = 5.00$), indicative of competitive binding to vanilloid receptors.

4 The stimulation of VO_2 produced by $0.2 \mu M$ PPAHV decreased, but was not completely abolished, after repeated infusion of PPAHV (change in VO_2 , first infusion, $0.66 \pm 0.18 \mu mol g^{-1} h^{-1}$; sixth infusion, $0.29 \pm 0.08 \mu mol g^{-1} h^{-1}$, $P < 0.05$), an acute tachyphylactic response not previously seen with the repeated infusion of other vanilloid analogues. Conversely, the PP response to repeated PPAHV infusion increased (ΔPP , first infusion, 5.8 ± 0.7 mmHg; sixth infusion, 9.0 ± 0.6 mmHg, $P < 0.05$).

5 In conclusion, PPAHV produces vasoconstriction and a biphasic effect on VO_2 in the perfused rat hindlimb very similar to that induced by naturally occurring vanilloids. Both effects are blocked by the competitive antagonist capsazepine. Since, the metabolic response to low concentrations of PPAHV (stimulation of VO_2) undergoes tachyphylaxis, the present data suggest that PPAHV desensitizes putative vanilloid receptors in the hindlimb.

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Abbreviations: Δ , change; PP, perfusion pressure; PPAHV, 12-phenylacetate 13-acetate 20-homovanillate; P_{VO_2} , venous oxygen partial pressure; RTX, resiniferatoxin; VO_2 , oxygen consumption

Introduction

The nociceptive neuron stimulant capsaicin produces a refractory state of desensitization with prolonged or repeated administration (tachyphylaxis). This property has promoted intense interest in its possible use as a non-steroidal analgesic and anti-inflammatory agent (Maggi, 1992; Dray & Urban, 1996). Recent attention has focused on other natural 'vanilloid' molecules including resiniferatoxin (RTX), an ultrapotent vanilloid analogue that shows high potency and a greater efficacy for inducing desensitization than for causing pain (Szallasi *et al.*, 1996; Szallasi & Blumberg, 1996; Appendino & Szallasi, 1997). However, the promotion of RTX as a therapeutic agent has been hampered by lack of availability since *Euphorbia resinifera* is a protected species. More recently, the development of synthetic phorbol-based vanilloids has yielded encouraging compounds such as phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPAHV), which is only mildly noxious and is devoid of

unwanted side effects at doses that protect against neurogenic inflammation (Appendino *et al.*, 1996).

Vanilloid-like properties of PPAHV have been demonstrated in recent radioligand binding (Appendino *et al.*, 1996) and patch clamp studies (Liu *et al.*, 1998). However, there have been very few studies examining its functional vanilloid-like effects in whole tissue preparations. In the perfused rat hindlimb, several natural vanilloid analogues have been shown to produce unique metabolic and vascular effects mediated by pharmacologically distinct (putative) vanilloid receptor subtypes (VN_1 and VN_2) (Colquhoun *et al.*, 1995; Griffiths *et al.*, 1996). Using the perfused rat hindlimb as a functional assay system, the present study examines the actions of PPAHV on resting muscle oxygen consumption (VO_2) and vascular resistance (determined by perfusion pressure, PP). In addition, the effects of repeated infusion of PPAHV were studied to determine the desensitizing properties of this agent in muscle. We provide solid evidence that PPAHV has strong vanilloid-like effects in this preparation and promotes rapid tachyphylaxis where other natural vanilloid analogues have failed to do so in our previous studies.

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Methods

All experimental procedures used in this study were approved by the University of Tasmania Animal Ethics Committee under the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (Australian Government, 1990).

Male 180–200 g hooded Wistar rats were housed at $21 \pm 1^\circ\text{C}$ under a 12 h light:dark cycle and fed a commercial rat chow diet containing 21.4% protein, 4.6% lipid, 68% carbohydrate and 6% crude fibre with added vitamins and minerals. Water was supplied *ad libitum*.

Hindlimb perfusion

Animals were anaesthetized with pentobarbitone sodium (60 mg kg^{-1}) and their left hindlimbs perfused according to the method described previously (Colquhoun *et al.*, 1988). In brief, flow was isolated to the left hindlimb by the cannulation of the abdominal aorta, posterior to the renal vessels, and the ligation of the tail, right common iliac and cutaneous blood vessels. The hindlimb was perfused under constant flow conditions (4 ml min^{-1}) with a modified Krebs-Ringer bicarbonate buffer containing 8.3 mM glucose, 1.27 mM CaCl_2 and 2% bovine serum albumin (Fraction V). All perfusions were conducted at 25°C and the perfusate was continually gassed with carbogen (95% $\text{O}_2/5\% \text{CO}_2$) to ensure a constant arterial PO_2 . The oxygen content of the venous effluent (venous oxygen partial pressure, P_{VO_2}) was measured continuously by directing outflow from the cannulated vena cava through an in-line 0.5 ml Clark-type oxygen electrode. Perfusion pressure (PP) was monitored by means of a pressure transducer adjoining the cannulated abdominal aorta.

The method of calculation of VO_2 has been described previously (Colquhoun *et al.*, 1988). Values for VO_2 calculation and PP were taken only after steady state conditions were obtained either under basal or drug-induced conditions.

Agent infusion

The infusion of various agents into the hindlimb commenced only after steady state VO_2 and PP had been reached. All agents were either freshly prepared before each experiment, or prepared and then stored at 4°C if chemically stable. Due to the lipophilic nature of vanilloids and their affinity for silicon-based tubing, PPAHV was infused using a syringe pump (Model 2620, Harvard apparatus, U.S.A.) driving a 1.0 ml glass syringe (SGE, Australia) equipped with teflon tubing. All other agents were infused with a second pump (Model 355, Sage instruments, Orion Research Inc., U.S.A.) using an identical 1.0 ml glass syringe and teflon tubing.

Concentration-response curves for PPAHV were constructed in a cumulative manner with at least two low (nanomolar) and two high (micromolar) concentrations. Each concentration was infused only after VO_2 and PP had reached steady state with the preceding concentration. In experiments using capsazepine ($10 \mu\text{M}$), the antagonist was first infused alone for approximately 5 mins, then co-infused with increasing concentrations of PPAHV.

In further experiments, PPAHV ($0.2 \mu\text{M}$) was infused repeatedly (six times) to determine the ability of PPAHV to cause acute desensitization in this preparation. On each occasion, the drug was infused for a sufficient length of time to achieve steady state VO_2 and PP responses (10–12 min).

Sufficient time (approximately 5 min) was also allowed in between each infusion to allow VO_2 and PP to return to basal (pre-stimulus) values.

Drugs and chemicals

Capsaicin was purchased from Sigma-Aldrich Chemical Co and capsazepine from Research Biochemicals International (U.S.A.). Phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPAHV) was a generous gift from Dr Giovanni Appendino (University of Torino, Torino, Italy; commercially available from Alexis Corporation, California, U.S.A.). Capsazepine was dissolved in ethanol (70–80%), as was PPAHV (50%). All other chemicals were of analytical grade.

Data and statistical analysis

The pK_b for capsazepine was determined from the perfusion pressure concentration response curves for PPAHV alone and in the presence of capsazepine (Figure 1) according to the Gaddum-Schild equation: $\text{pK}_b = \log (\text{CR} - 1) / [\text{antagonist}]$, where CR is the concentration ratio.

With the exception of the typical tracings, all data are presented as mean \pm s.e.mean. Statistical analysis was performed using one-way repeated measures analysis of variance (ANOVA) with *post-hoc* Student-Newman-Keuls analysis. $P < 0.05$ was considered statistically significant.

Results

Figure 1 shows concentration-response curves for the effect of the synthetic vanilloid analogue PPAHV on VO_2 and PP in

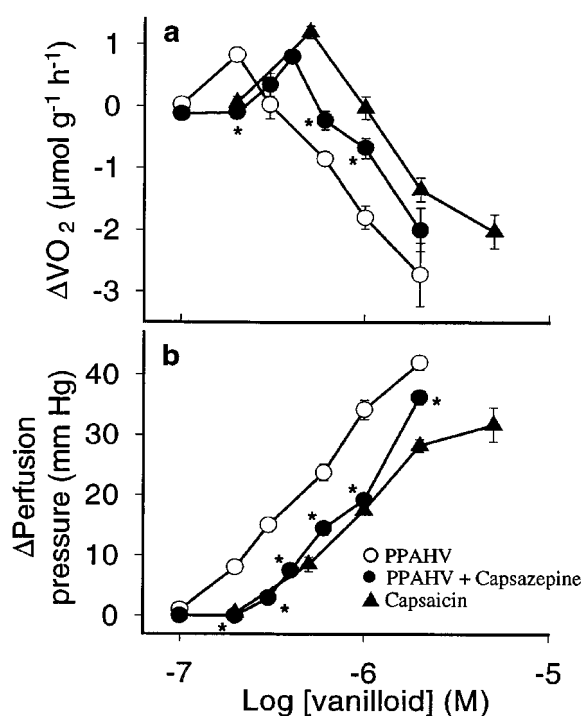


Figure 1 Concentration-response curves for (a) oxygen consumption (VO_2) and (b) perfusion pressure (PP) in the perfused rat hindlimb for 12-phenylacetate 13-acetate 20-homovanillate (PPAHV) alone and in the presence of $10 \mu\text{M}$ capsazepine, compared to previously published data for capsaicin (Griffiths *et al.*, 1996). Values are mean \pm s.e.mean of 4–5 experiments. * $P < 0.05$, response significantly different from PPAHV alone (ANOVA).

the perfused rat hindlimb. PPAHV produced a biphasic effect on VO_2 , in association with concentration-dependent vasoconstriction, that is also characteristic of the infusion of other natural vanilloids into this preparation. Maximum stimulation of VO_2 was observed at $0.2 \mu\text{M}$ PPAHV (ΔVO_2 , $0.83 \pm 0.06 \mu\text{mol g}^{-1} \text{h}^{-1}$) and was accompanied by a mild increase in PP (ΔPP , $8.0 \pm 1.1 \text{ mmHg}$). Higher (micromolar) concentrations inhibited VO_2 and caused pronounced vasoconstriction. At the highest concentration of PPAHV tested ($2 \mu\text{M}$), VO_2 and PP changes were $-2.73 \pm 0.51 \mu\text{mol g}^{-1} \text{h}^{-1}$ and $42.0 \pm 1.2 \text{ mmHg}$, respectively.

In the presence of capsazepine ($10 \mu\text{M}$), concentration-response curves for PPAHV-induced changes in VO_2 and PP were shifted (parallel) to the right (Figure 1). Maximum stimulation of VO_2 (similar to that observed in the absence of capsazepine) was attained using $0.4 \mu\text{M}$ PPAHV (change in VO_2 , $0.80 \pm 0.10 \mu\text{mol g}^{-1} \text{h}^{-1}$). However, the highest observed PP response to PPAHV was significantly ($P < 0.05$) lowered by capsazepine (change in PP, $36.3 \pm 1.1 \text{ mmHg}$), although this could be overcome by increasing the concentration of PPAHV. Using the Gaddum-Schild equation, the estimated pK_b for capsazepine in the present studies was five.

Comparison of the VO_2 and PP concentration response curves for PPAHV and those for capsaicin reveals that PPAHV is slightly more potent than the natural analogue (Figure 1). This could not be confirmed by estimating the half-maximal effect concentration (EC_{50}) for these ligands as maximum responses for the PPAHV-induced PP change and inhibition of VO_2 were not observed at the concentrations used.

A typical tracing of the effects of repeated infusion of $0.2 \mu\text{M}$ PPAHV on venous PO_2 and PP is shown in Figure 2. PPAHV rapidly induced a fall in venous PO_2 (thus increasing VO_2) to a new steady state value, and increased PP. The fall in venous PO_2 gradually diminished with successive infusions of PPAHV, while PP increased further. Mean data from five experiments are shown in Figure 3. The first PPAHV infusion produced a typical stimulation of VO_2 (change in VO_2 , $0.66 \pm 0.18 \mu\text{mol g}^{-1} \text{h}^{-1}$). However, a decrease in the VO_2 stimulatory effect was observed with subsequent infusions which achieved statistical significance ($P < 0.05$) with the fourth ($0.29 \pm 0.09 \mu\text{mol g}^{-1} \text{h}^{-1}$), fifth ($0.31 \pm 0.07 \mu\text{mol g}^{-1} \text{h}^{-1}$) and sixth infusions ($0.29 \pm 0.08 \mu\text{mol g}^{-1} \text{h}^{-1}$; Figure 3a). In contrast, repeated infusion of PPAHV was associated with a significant ($P < 0.05$) increase in PP (first infusion, 5.8 ± 0.7 ; fourth infusion, 8.6 ± 0.05 ; fifth infusion, 9.0 ± 0.7 ; and sixth infusion, $9.0 \pm 0.7 \text{ mmHg}$, Figure 3b).

Discussion

A variety of naturally occurring vanilloids, including capsaicin, RTX and piperine, produce a biphasic effect on hindlimb VO_2 , namely stimulation at low concentrations and inhibition at higher concentrations, with both actions associated with vasoconstriction (Eldershaw *et al.*, 1994; Colquhoun *et al.*, 1995; Griffiths *et al.*, 1996; 1998). Similarly, in the present study, submicromolar concentrations of the synthetic vanilloid agonist PPAHV stimulated VO_2 which was associated with mild vasoconstriction, whereas higher (micromolar) concentrations of the agonist inhibited VO_2 and produced marked vasoconstriction. Thus, PPAHV produced the same metabolic and vascular effects that other natural analogues produce in this functional assay preparation. In addition, we demonstrated for the first time, the powerful desensitizing properties of PPAHV which, unlike other

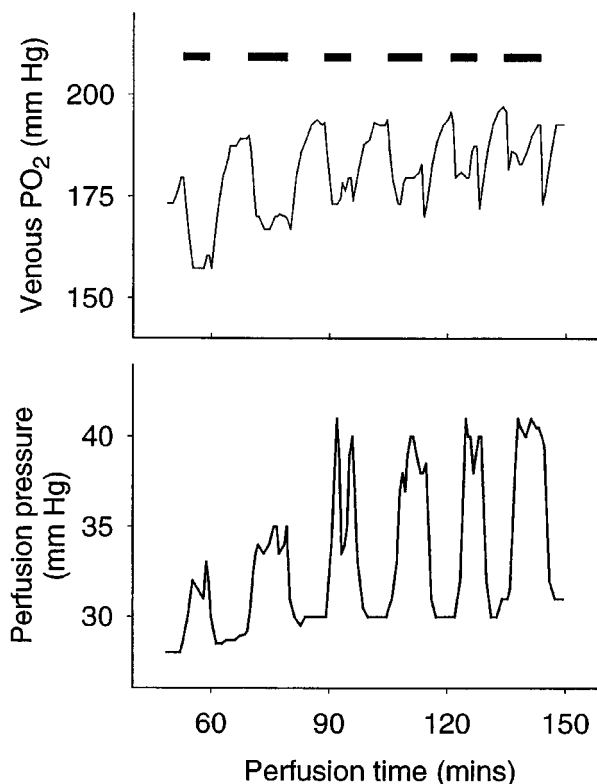


Figure 2 Typical tracing for the actions of 12-phenylacetate 13-acetate 20-homovanillate (PPAHV; $0.2 \mu\text{M}$) on venous PO_2 and perfusion pressure (PP) in the perfused rat hindlimb. The first infusion of PPAHV caused a strong reduction in venous PO_2 (and hence stimulation of VO_2) and a mild increase in hindlimb PP reflecting a vasoconstrictor effect. Removal of PPAHV resulted in the return of venous PO_2 and PP to basal levels. With subsequent infusion of the same concentrations of PPAHV, the effect on venous PO_2 was markedly reduced while that for PP was enhanced.

vanilloid agonists, caused pronounced acute tachyphylaxis with repeated infusion.

It is very likely that PPAHV produces its effects by binding to specific vanilloid receptors (Caterina *et al.*, 1997), since the vanilloid antagonist capsazepine caused a parallel shift to the right of the concentration-response curves for PPAHV, with little change to the maximum. The competitive inhibition of PPAHV by capsazepine was very similar to that observed for capsaicin (Griffiths *et al.*, 1996), suggesting that all three compounds interact with the same vanilloid recognition sites. The pK_b for capsazepine estimated from the concentration-response curves for PPAHV in Figure 1 ($\text{pK}_b = 5.00$) was consistent with our previously published findings when capsaicin was used as the agonist ($\text{pK}_b = 5.07$, Griffiths *et al.*, 1996). In addition, the potency of PPAHV in this preparation, relative to that of capsaicin and resiniferatoxin (RTX) (Eldershaw *et al.*, 1994), is in excellent agreement with the affinity of this compound for vanilloid receptors previously determined in radioligand binding assays (Szallasi *et al.*, 1996).

The biphasic effect of PPAHV and other vanilloids on perfused muscle VO_2 is an interesting, but unexplained, phenomenon. One possibility is that vanilloids bind to their muscle receptors in an allosteric fashion such as that seen for RTX and other vanilloid ligands which produce a biphasic effect on [^3H]-RTX binding (an initial increase in binding followed by inhibition) on dorsal root ganglion (DRG) vanilloid receptors (Szallasi *et al.*, 1993; Acs & Blumberg, 1994). That is, the biphasic competition curves for capsaicin

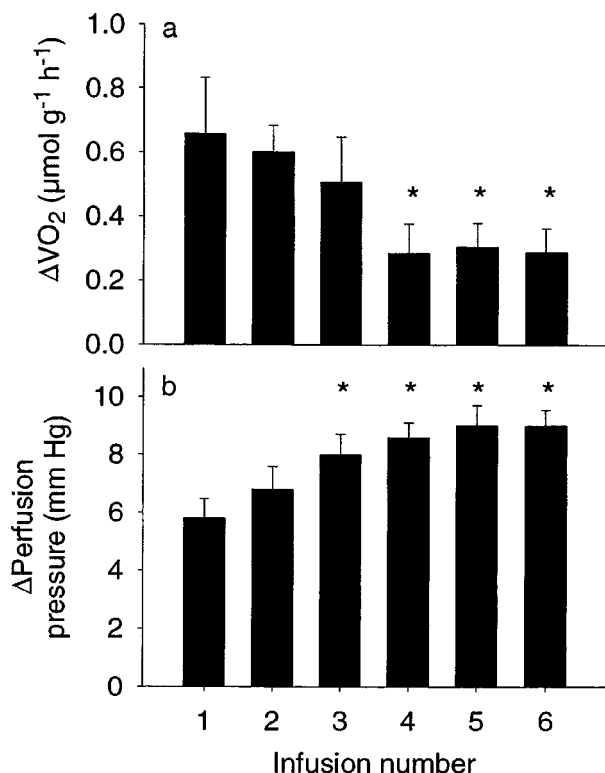


Figure 3 Effect of six repeated infusions of 12-phenylacetate 13-acetate 20-homovanillate (PPAHV; $0.2 \mu\text{M}$) on rat hindlimb (a) change in oxygen consumption (VO_2), (b) change in perfusion pressure (PP). Values are the mean \pm s.e. mean of five experiments. * $P < 0.05$, response significantly different from first infusion (one-way repeated measures ANOVA).

and RTX observed in [^3H]-RTX binding studies show striking resemblance to the biphasic nature of VO_2 concentration-response curves for capsaicin and RTX in the perfused rat hindlimb (Colquhoun *et al.*, 1995; Griffiths *et al.*, 1996; 1998), suggesting that vanilloids may bind to hindlimb receptors in a cooperative (allosteric) fashion. If this hypothesis were correct, it might be expected that PPAHV would produce monophasic curves for VO_2 in the hindlimb preparation since it produces monophasic competition curves in radioligand binding studies on DRG neurons (non-cooperativity) (Szallasi *et al.*, 1996). However, the data presented in Figure 1 demonstrate that this is not the case and provides preliminary evidence that the biphasic VO_2 curves observed with vanilloid infusion into this preparation are not the result of positive cooperativity.

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We have previously shown that acute tachyphylaxis with repeated infusion of natural vanilloid analogues could not be achieved in this preparation (Eldershaw *et al.*, 1994), and systemic capsaicin pretreatment was required to cause a reduced response to infused capsaicin (Griffiths *et al.*, 1998). In the present study, the VO_2 response to a submicromolar concentration of PPAHV declined with repeated infusion, without the requirement of chronic vanilloid pretreatment. The concentration of the drug used to achieve this was more than 10 fold lower than that used to eliminate rapidly activating currents in rat trigeminal ganglia (Liu *et al.*, 1998), and the observed dissociation constant for PPAHV (Szallasi *et al.*, 1996). The tachyphylaxis observed in the perfused hindlimb is not likely to be related to a decrease in the viability of the preparation since the vasoconstrictor response increased.

The PP response was enhanced, rather than attenuated, by repeated infusion of PPAHV and could be regarded as evidence of sensitization rather than desensitization. However, we recently demonstrated that while systemic capsaicin pretreatment can abolish the stimulation of VO_2 by vanilloid infusion into the hindlimb, the vasoconstrictor response was enhanced (Griffiths *et al.*, 1998). It is unlikely that these vascular resistance changes can explain the observed decrease in VO_2 responsiveness (tachyphylaxis) since the elevated PP response was first observed after the third infusion when the VO_2 response was normal (Figure 3). Conversely, a similar elevated PP response was observed with the fourth infusion, but the VO_2 response was dramatically reduced.

In conclusion, the RTX-like synthetic vanilloid PPAHV produced characteristic capsaicin-like VO_2 and PP responses in the perfused rat hindlimb, and was able to induce acute tachyphylaxis preparation with repeated infusion. The ability of PPAHV to cause tachyphylaxis in this preparation, where other natural vanilloids cannot, is not surprising given that its structure is based on the ultrapotent vanilloid RTX, a compound renowned for its powerful desensitizing properties. The mechanism of this desensitization is not clear from these studies but may be related to neuropeptide depletion and/or internalization of neuropeptide or vanilloid receptors. Despite the lack of knowledge regarding the mechanism of tachyphylaxis, PPAHV and more recently described phorbol-based compounds (Szallasi *et al.*, 1999) remain exciting prospects for the development of anti-inflammatory-analgesic agents with few side effects.

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