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A novel agonist, phorbol 12-phenylacetate 13-acetate 20-homovanillate, abolishes positive cooperativity of binding by the vanilloid receptor

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Abstract

Capsaicin binds to a specific recognition site, referred to as the vanilloid receptor, which it shares with the natural, ultrapotent agonist resiniferatoxin and with the competitive antagonist capsazepine. Upon binding to its receptor, capsaicin opens a cation channel leading to Ca^{2+} influx. The binding of capsaicin or resiniferatoxin by the vanilloid receptor follows a sigmoidal saturation curve, indicative of positive cooperativity. The biological significance of this positive cooperative behaviour is unknown, as is the mechanism responsible for it. We have developed a novel ligand, phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPAHV), which binds to cultured rat sensory neurons (with a K_i of 3.1 ± 0.4 μM), and induces Ca^{2+} uptake by them (with an ED_{50} of 1.8 ± 0.3 μM) with similar affinities and in a non-cooperative manner (Hill coefficients are 0.99 and 1.06 for binding and Ca^{2+} uptake, respectively). The behaviour of PPAHV thus contrasts with resiniferatoxin or capsaicin not only in the lack of cooperativity but also in the relative potencies for resiniferatoxin binding versus Ca^{2+} uptake (resiniferatoxin is less potent and capsaicin is more potent for induction of Ca^{2+} uptake than for binding). In further experiments in which the concentration of [^{3}H]resiniferatoxin was varied, 1 μM PPAHV likewise reduced the cooperativity index that characterizes resiniferatoxin binding to rat spinal cord membranes from 2.3 ± 0.1 to 1.1 ± 0.2; in parallel experiments, neither capsaicin nor capsazepine (both at a concentration of 2 μM) affected binding cooperativity. Moreover, PPAHV (1 μM) turned the bi-phasic dissociation curve of resiniferatoxin into a monophasic curve, eliminating the second, slow-dissociation phase. The present results suggest that positive cooperativity is a ligand-induced feature rather than an inherent property of vanilloid receptors. A comparison of the spectrum of biological activity of ligands which bind to vanilloid receptors with different degrees of cooperativity may provide an approach to explore the functional significance of this binding behaviour.

Keywords: Vanilloid receptor; Binding cooperativity; Resiniferatoxin; Capsaicin; PPAHV (phorbol 12-phenylacetate 13-acetate 20-homovanillate)

1. Introduction

Capsaicin, the pungent principle in hot pepper, has long been in use to identify a both functionally and neurochemically diverse subpopulation of primary sensory neurons (Szolcsanyi, 1984; Buck and Burks, 1986; Holzer, 1991 for reviews). Excitation by capsaicin of these neurons is followed by a refractory state which is traditionally termed desensitization (Jancso, 1968). High capsaicin doses may also cause gross neurotoxicity, especially when given to newborn animals (Jancso et al., 1977). Capsaicin is already in use as a counterirritant to relieve neuropathic pain (Carter, 1991 for review); animal experimentation suggests an even greater therapeutic potential for capsaicin desensitization as a non-narcotic, non-steroid antiinflammatory and pain-killer agent (Szolcsanyi, 1991; Maggi, 1992; Szallasi and Blumberg, 1993a for reviews).

The fairly strict structure-activity requirements for capsaicin-like activity (Szolcsanyi and Jancso-Gabor, 1975) predicted the existence of a 'capsaicin receptor'; however, despite several efforts, radiolabelled capsaicin failed to identify a receptor. Recently, resiniferatoxin, an extremely irritant diterpene ester isolated from several species of the genus *Euphorbia* (Hergenhahn et al., 1975), turned out to function as an ultrapotent analog of capsaicin (Szallasi and

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Blumberg, 1990b; Blumberg et al., 1993 for reviews). This discovery led to the synthesis of a radioligand, [^{3}H]resiniferatoxin, which, on the one hand, demonstrated the existence of specific, saturable binding sites shared by resiniferatoxin, capsaicin, and capsazepine (Szallasi and Blumberg, 1990a; Szallasi, 1994), an antagonist of resiniferatoxin as well as capsaicin actions (Bevan et al., 1992), and, on the other hand, made the visualization of capsaicin-sensitive neurons possible by an autoradiographic approach (Szallasi et al., 1994). Receptors are preferably named after their endogenous ligand(s). Until an endogenous ligand of the capsaicin receptor is identified, this receptor appears to be best described as the 'vanilloid receptor', given the essential role of a (homo)vanillyl moiety in the biological activity of the xenobiotics it recognizes (Szallasi and Blumberg, 1990b; Blumberg et al., 1993; Szallasi, 1994).

Receptors containing intrinsic ion channels are multisubunit. The vanilloid receptor is thought to contain a non-selective cation channel (Bevan and Szolcsanyi, 1990; James et al., 1993 for reviews) and its radiation inactivation size (Szallasi and Blumberg, 1991) is in accord with an oligomeric structure. Such a structure is a prerequisite for cooperative (either positive or negative) binding behaviour. Thus, it is hardly unexpected that the vanilloid receptor turned out to bind ligands in a positive cooperative fashion (Szallasi et al., 1993). The biological role for this positive cooperative behaviour is unknown, as is the mechanism responsible for it. In general, there are two basic models to explain positive binding cooperativity, both of which assume the existence of a receptor oligomer with identical binding sites. The induced-fit model (Koshland et al., 1966) assumes progressive, cumulative transitions in affinity, whereas in the concerted model (Monod et al., 1965) the transitions are all or none. In this work, by examining the binding mechanism of naturally occurring as well as synthetic vanilloids, we attempt to determine the model which best applies for the vanilloid receptor. Moreover, we try to identify the relationship between binding cooperativity of a given vanilloid and a resulting functional response, i.e. Ca^{2+} uptake by rat dorsal root ganglion neurons cultured in vitro.

2. Materials and methods

2.1. Membrane preparation

Membranes for binding experiments were obtained according to a published procedure (Szallasi and Blumberg, 1990a). Briefly, the cervical and thoracic segments of the spinal cord were quickly removed from female Sprague-Dawley rats (200–250 g) killed by decapitation under CO_2 anesthesia, and collected into ice-cold buffer A (pH 7.4), which contained (in mM) NaCl 5.8, KCl 5, $CaCl_2$ 0.75, $MgCl_2$ 2, sucrose 137, and Hepes 10. Tissues were disrupted with the aid of a Polytron tissue homogenizer in the same buffer; homogenates were first centrifuged for 10 min at $1000 \times g$ (4°C), and then the resulting supernatants were further centrifuged for 30 min at $35000 \times g$ (4°C). The pellets from the second centrifugation were resuspended in buffer A to yield an estimated concentration of 1 mg protein per ml; the membrane suspensions were aliquoted and then stored at −80°C until assayed. The protein concentrations of the membrane suspensions were determined using a Bio Rad kit.

2.2. Cell cultures

For rat dorsal root ganglia cultures (Wood et al., 1988; Acs et al., 1995a), the spinal columns were removed aseptically and dorsal root ganglia from all levels of the spinal cord were dissected out and collected in Hank's Balanced Salt Solution without Ca^{2+}, Mg^{2+} and phenol red (HBSS), containing 0.5% heat-inactivated fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA), 1 mM Na-pyruvate, 25 mM Hepes and antibiotics. Ganglia were digested with 0.125% collagenase (Sigma, St. Louis, MO, USA) in HBSS for 90 min at 37°C, then for a further 90 min in a fresh collagenase solution. Ganglia were washed twice with Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 25 mM Hepes and 1 mM Na-pyruvate, and were then triturated through a flame-polished Pasteur pipette to form a single cell suspension. The cells were pelleted through a cushion of DMEM containing 15% fatty acid free bovine serum albumin (Sigma, St. Louis, MO, USA) to remove myelin debris. Cells were then washed 3 times with DMEM; they were resuspended in the same medium; and the number of viable cells was determined. Finally, cells were plated in Multiscreen-DV 96-well filtration plates (Millipore, Marlborough, MA, USA) at a density of 5000 cells per well in 100 μl medium and were immediately used for $^{45}Ca^{2+}$ uptake or [^{3}H]resiniferatoxin binding experiments, respectively.

2.3. Ca^{2+} uptake assays

For $^{45}Ca^{2+}$ uptake (Wood et al., 1988; Acs et al., 1995a), dorsal root ganglia neurons were incubated in serum-free DMEM (containing 1.8 mM $CaCl_2$) in the presence of 0.25 mg/ml bovine serum albumin (included in order to stabilize the compounds in the aqueous solution), 1 μCi/ml $^{45}Ca^{2+}$ (23.55 mCi/mg; DuPont-New England Nuclear, Boston, MA, USA), and increasing concentrations of the different compounds for 20 min at 37°C. Cells were then washed 6 times with ice-cold HBSS by filtration using a MultiScreen Vacuum Manifold (Millipore, Marlborough, MA, USA). Filters were dried under a heat lamp, and the radioactivity was determined by scintil-

lation counting. For each data point eight wells were assayed.

2.4. [^{3}H]Resiniferatoxin binding experiments

[^{3}H]Resiniferatoxin binding assays were performed using spinal cord membranes (Szallasi et al., 1993) and isolated dorsal root ganglia neurons (Acs et al., 1995a), respectively. For saturation binding, aliquots of the spinal cord preparations (40–50 μg protein) were incubated in triplicate with increasing concentrations (6–400 pM) of [^{3}H]resiniferatoxin (37 Ci/mmol; synthesized by the Chemical Synthesis and Analysis Laboratory, NCI-FCRDC, Frederick, MD, USA) in 500 μl of buffer A containing 0.25 mg/ml bovine serum albumin (Cohn fraction V, Sigma, St. Louis, MO, USA). Non-specific binding was defined as that occurring in the presence of 100 nM non-radioactive resiniferatoxin (LC Laboratories, Woburn, MA, USA). For competition experiments, membranes were incubated with 20 pM [^{3}H]resiniferatoxin, the approximate K_d from the saturation studies (see Results); competing ligands were added using 1 : 3 dilutions; stock solutions were made up in ethanol and were then diluted in buffer A containing 10 mg/ml bovine serum albumin. The final concentration of the organic solvents in the assay mixture never exceeded 0.1% (v/v); such concentrations did not have any detectable effects on [^{3}H]resiniferatoxin binding. Assay mixtures were kept on ice while the additions were made. The binding reaction was initiated by transferring the assay tubes into a shaking water bath (37°C) and then terminated following a 60 min incubation by cooling the assay mixtures on ice. Non-specific binding was then reduced by adding 100 μg of bovine α_1-acid glycoprotein (Sigma, St. Louis, MO, USA), a plasma protein that binds resiniferatoxin even at 0°C (Szallasi et al., 1992), to each tube. Bovine α_1-acid glycoprotein functions by sequestering free resiniferatoxin (Szallasi et al., 1992). Because the non-specifically bound resiniferatoxin in the membranes is in equilibrium with free resiniferatoxin, the addition of α_1-acid glycoprotein leads to a net shift of the non-specifically bound resiniferatoxin out of the membranes (Szallasi et al., 1992). However, because of the undetectably slow off-rate at 0°C of the receptor-bound resiniferatoxin (Szallasi and Blumberg, 1993b), specific binding is not affected. Bound and free [^{3}H]resiniferatoxin were separated by pelleting the membranes in a Beckman 12 microfuge (Beckman Instruments AB, Stockholm, Sweden) and then quantitated by scintillation counting.

[^{3}H]Resiniferatoxin binding to intact, isolated dorsal root ganglia neurons (Acs et al., 1995a) was determined with some modifications of the above protocol: as an assay buffer, 250 μl of DMEM containing 0.5 mg/ml bovine serum albumin was used; after the termination of the binding reaction, cells were washed by filtration 4 times with DMEM (200 μl per well) supplemented with 0.5 mg/ml α_1-acid glycoprotein using a MultiScreen Vacuum Manifold (Millipore, Marlborough, MA, USA); filters dried under a heat lamp were punched out into scintillation vials, and the bound radioactivity was determined by scintillation counting.

2.5. Analysis of data

Data were analyzed either by the collection of computer programs of McPherson (1985), collectively referred to as KELL (Biosoft, Cambridge, UK), or by a computer program which fits the allosteric Hill equation (Endrenyi et al., 1975) to the measured values (FitP; Biosoft, Cambridge, UK). Binding data from competition experiments were also analyzed by a computer fit to the modified Hill equation (Davis et al., 1977) as described previously (Szallasi et al., 1993; Acs et al., 1995a).

2.6. Materials

The synthesis and spectroscopic characterization of phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPAHV) will be described elsewhere (Appendino, Cravotto, Palmisano, Annunziata and Szallasi, submitted). Briefly, phorbol 20-trityl ether was esterified selectively at O–13 (Ac_2O, triethylamine, tetrahydrofuran) and then at O–12 (phenylacetic acid, dicyclohexylcarbodiimide, demethylaminopyridine), deprotected ($HClO_4$), and esterified at O–20 with MEM-homovanillic acid (dicyclohexylcarbidiimide, dimethylaminopyridine). Deprotection of the phenolic ether was achieved with $SnCl_4$ in tetrahydrofuran. The overall yield, starting from phorbol 20-trityl ether, was 38%. The stability of PPAHV in aqueous solution (37°C; over a period of 24 h) was checked by HPLC: no degradation was detected.

Capsazepine was purchased from RBI (Natick, MA, USA). All the other chemicals including capsaicin were obtained from Sigma (St. Louis, MO, USA), unless indicated otherwise, and were of the highest quality available.

3. Results

3.1. Characterization of binding of phorbol 12-phenylacetate 13-acetate 20-homovanillate to rat spinal cord membranes

In competition assays performed in the presence of 20 pM [^{3}H]resiniferatoxin, the approximate K_d from the saturation binding experiments (see below), phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPAHV) displayed a K_i value of 0.6 ± 0.1 μM (three determinations, mean $\pm$ S.E.M.; Fig. 1). In parallel experiments, non-radioactive resiniferatoxin inhibited specific binding of 20 pM [^{3}H]resiniferatoxin by 50% at a concentration of 100 pM (Fig. 1). As predicted by the modified Hill equation, an initial 100% enhancement by non-radioactive resiniferatoxin of

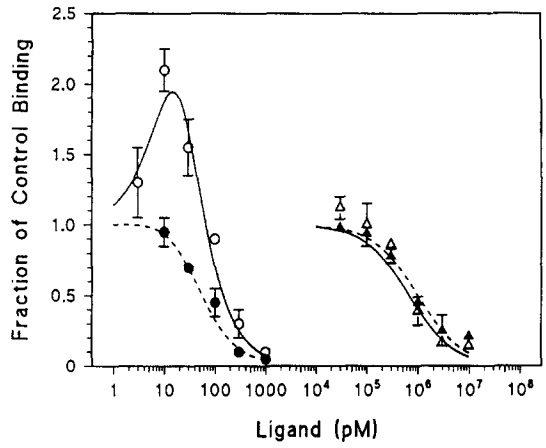

Fig. 1. Enhancement/inhibition of specific [^{3}H]resiniferatoxin binding to rat spinal cord membranes by non-radioactive resiniferatoxin (circles) or phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPAHV) (triangles) as determined at 7 pM (open symbols) and 20 pM (closed symbols) concentrations of the labeled compound. These concentrations correspond to approximately 10% and 50% receptor occupancy, respectively. Theoretical curves were generated using the modified Hill equation. As shown, affinity for resiniferatoxin, 20 pM; affinity for PPAHV, 500 nM; cooperativity index for resiniferatoxin, 2.1; and cooperativity index for PPAHV, 1.0. Points are mean values of triplicate assays from a single experiment; error bars represent S.E.M. Two additional experiments gave similar results.

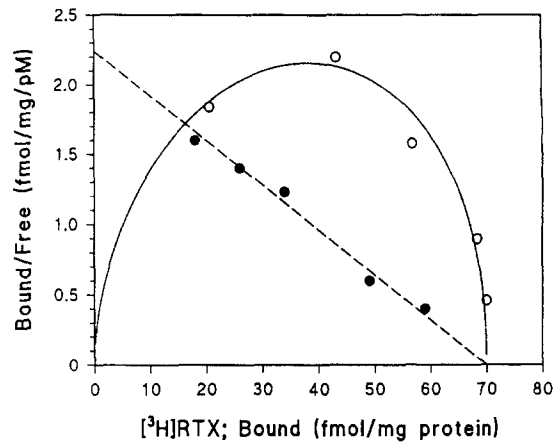

Fig. 2. Scatchard plots of specific [^{3}H]resiniferatoxin binding to rat spinal cord membranes in the absence (open circles) or presence (closed circles) of 1 μM phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPAHV). The theoretical curve for control binding was generated using the apparent K_d (17 pM), cooperativity index (2.2) and B_{max} (70 fmol/mg protein) values from a fit to the allosteric Hill equation. For binding in the presence of 1 μM PPAHV, the line was fitted using the curvilinear regression program LIGAND. Note that PPAHV turns the convex Scatchard plot, which is characteristic of positive binding cooperativity, into a linear plot. Data are from a single experiment; for control conditions, eight additional experiments, and for PPAHV, five additional experiments yielded similar results.

specific [^{3}H]resiniferatoxin binding (7 pM) was seen at approximately 10% fractional receptor occupancy (see below), preceding inhibition (Fig. 1); this binding behaviour is characteristic of positive cooperativity. Addition of non-radioactive resiniferatoxin did not modify the apparent concentration of free [^{3}H]resiniferatoxin in the assay mixtures. Interestingly, in similar experiments PPAHV failed to enhance specific resiniferatoxin binding (Fig. 1). This latter finding implies that PPAHV, unlike resiniferatoxin, binds to rat spinal cord membranes in a non-cooperative fashion. To further verify this hypothesis, specific binding of increasing concentrations of [^{3}H]resiniferatoxin was analyzed in the absence or presence of 1 μM PPAHV, the concentration that inhibited [^{3}H]resiniferatoxin binding by 50% in the competition assays (Fig. 1). As expected, 1 μM PPAHV (L) reduced the affinity of the spinal vanilloid receptor for [^{3}H]resiniferatoxin from 21 ± 1 pM (K_d) to 46 ± 3 pM (K_{app}) without a measurable change in maximal receptor density (58 ± 3 fmol/mg protein in the absence and 62 ± 5 fmol/mg protein in the presence of 1 μM PPAHV, respectively; means ± S.E.M. of six determinations; Fig. 2; Table 1). From this shift in affinity, a K_i value of 0.5 ± 0.1 μM can be calculated, using the equation $K_i = L/(K_{app}/K_d - 1)$. Thus, the affinities of PPAHV to vanilloid receptors in rat spinal cord determined in two distinct protocols (0.6 μM and 0.5 μM) are in excellent agreement. 1 μM PPAHV, however, almost completely abolished the positive cooperativity that characterizes resiniferatoxin binding to rat spinal cord membranes: Hill numbers were 2.3 ± 0.1 and 1.1 ± 0.2 in the absence or presence of 1 μM PPAHV, respectively (six determinations, means ± S.E.M.; Fig. 2; Table 1). The Scatchard plots of the data (convex versus linear) demonstrate the dramatic effect of PPAHV on [^{3}H]resiniferatoxin binding (Fig. 2). In a similar experimental setup, neither capsaicin nor capsazepine affected binding cooperativity (Hill numbers were 2.2 ± 0.2 in control experiments and 2.4 ± 0.2 in the presence of 2 μM capsazepine or 1.9 ± 0.2 in the presence of 2 μM capsaicin, respectively; mean ± S.E.M.; three determinations; Fig. 3; Table 1).

3.2. Effect of phorbol 12-phenylacetate 13-acetate 20-homovanillate on the dissociation of [^{3}H]resiniferatoxin from rat spinal cord membranes

Dissociation of specifically bound [^{3}H]resiniferatoxin from rat spinal cord membranes follows complex kinetics

Table 1
Effects of 1 μM phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPAHV), 2 μM capsaicin or 2 μM capsazepine on parameters of specific [^{3}H]resiniferatoxin binding to rat spinal cord membranes

	Affinity (pM)	B_{max} (fmol/mg prot.)	Hill coefficient
Control	21 ± 1	58 ± 3	2.3 ± 0.1
PPAHV	46 ± 3	62 ± 5	1.1 ± 0.2
Capsaicin	41 ± 6	66 ± 4	1.9 ± 0.2
Capsazepine	40 ± 3	61 ± 4	2.4 ± 0.2

Mean ± S.E.M.; control, nine determinations; PPAHV, six determinations; capsaicin and capsazepine, three determinations each.

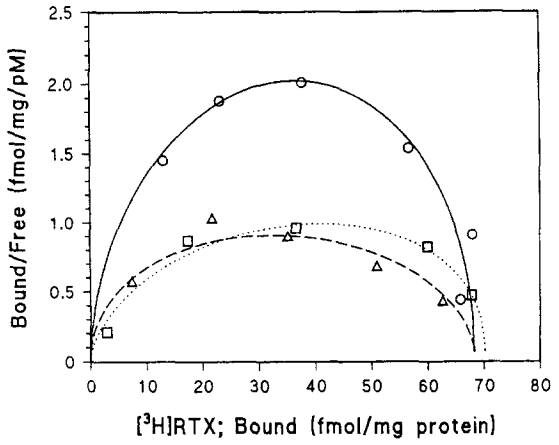

Fig. 3. Scatchard plots of specific [^{3}H]resiniferatoxin binding to rat spinal cord membranes in the absence (circles) or the presence of 2 μM capsaicin (triangles) or capsazepine (squares). Theoretical curves were generated using binding parameters from a fit to the allosteric Hill equation. As shown, apparent binding affinities and cooperativity indices are 17 pM and 2.15 under control conditions; 38 pM and 1.9 in the presence of 2 μM capsaicin; and 36 pM and 2.4 in the presence of 2 μM capsazepine, respectively. Points are from a single experiment. Replication of the experiment (under control conditions, 8 times; in the presence of capsaicin or capsazepin, twice) gave similar results.

with at least two phases, characterized by fast and slow off-rates, respectively (Fig. 4). The ratio between these two states depends on the fractional receptor occupancy by

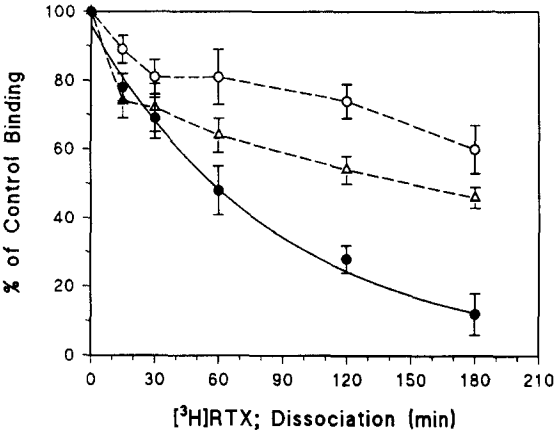

Fig. 4. Dissociation of specifically bound [^{3}H]resiniferatoxin from rat spinal cord membranes. Membranes were preincubated with 25 pM [^{3}H]resiniferatoxin in the absence (open circles) or presence (closed circles) of phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPAHV) or with 12 pM [^{3}H]resiniferatoxin (triangles). After steady-state binding had been achieved (60 min at 37°C), release of the bound ligand was initiated by adding 1000 μg of α_1-acid glycoprotein, a plasma protein that binds resiniferatoxin, to each tube to prevent the rebinding of the dissociated radioligand. If steady-state binding was achieved in the presence of PPAHV, the dissociation data could be fitted to the mono-exponential model, yielding a dissociation rate constant of 0.018 min^{-1}. If steady-state binding was achieved in the presence of [^{3}H]resiniferatoxin alone, dissociation curves were inconsistent with the mono-exponential model, indicating the existence of at least two distinct dissociation states. Points are mean values from a single experiment; error bars indicate S.E.M. of triplicate determinations. Two additional experiments yielded similar results.

[^{3}H]resiniferatoxin (Fig. 4) at the time (a 60 min preincubation at 37°C to achieve steady-state binding) when the dissociation was initiated by adding 1000 μg of α_1-acid glycoprotein, a plasma protein which binds resiniferatoxin, to prevent rebinding of the dissociated radioligand. When steady-state binding was achieved in the presence of 25 pM [^{3}H]resiniferatoxin and 1 μM PPAHV (specific binding is very similar to that determined in the presence of 12 pM [^{3}H]resiniferatoxin only), a monophasic dissociation curve was observed, lacking the second, slow-dissociation phase (Fig. 4). This contrasts with the bi-phasic dissocation curves observed using [^{3}H]resiniferatoxin alone at either 25 pM or 12 pM, respectively (Fig. 4). Neither capsaicin nor capsazepine changed the bi-phasic dissociation kinetics of [^{3}H]resiniferatoxin (not shown).

3.3. Comparison between binding of phorbol 12-phenylacetate 13-acetate 20-homovanillate to intact rat dorsal root ganglia neurons in culture and the resulting Ca^{2+} influx

PPAHV binds to cultured rat dorsal root ganglia neurons with an affinity of 3.1 $\pm$ 0.4 μM in a non-cooperative fashion (Hill coefficient is 1.06 $\pm$ 0.11; means $\pm$ S.E.M.; three determinations; Fig. 5). Uptake of $^{45}Ca^{2+}$ induced by PPAHV follows hyperbolic saturation kinetics, implying a non-cooperative response (Fig. 6). In fact, when fitted to the allosteric Hill equation, Ca^{2+} uptake data yield a cooperativity index close to unity (0.99 $\pm$ 0.17, mean $\pm$ S.E.M.; four determinations). Half-maximal Ca^{2+} uptake response occurs at a concentration of 1.8 $\pm$ 0.3 μM PPAHV (mean $\pm$ S.E.M.; four determinations; Fig. 6). Thus, the affinity of PPAHV to vanilloid receptors in cultured dorsal root ganglia neurons (3.1 μM) and its

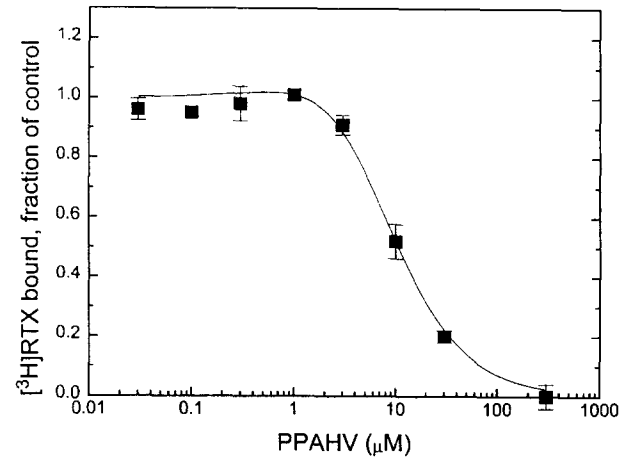

Fig. 5. Inhibition by phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPAHV) of specific [^{3}H]resiniferatoxin binding to intact, dissociated rat dorsal root ganglion neurons. The competition curve was fitted using the modified Hill equation. Data are from a representative experiment; points represent mean values from triplicate determinations; error bars indicate S.E.M. Two additional experiments gave similar results.

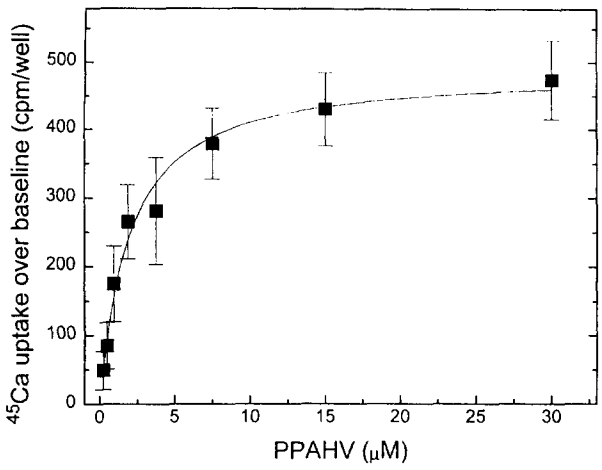

Fig. 6. Dose-response relations for phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPAHV)-induced $^{45}Ca^{2+}$ uptake by rat dorsal root ganglia neurons cultured in vitro. The line was fitted using the allosteric Hill equation. Points are mean values from assays done in triplicate; error bars indicate S.E.M. Two additional experiments gave similar results.

potency to induce Ca^{2+} uptake by them (1.8 μM) are in good agreement.

4. Discussion

Specific binding of [^{3}H]resiniferatoxin, an ultrapotent capsaicin analog (Szallasi and Blumberg, 1990a; Blumberg et al., 1993; Szallasi, 1994), has revealed marked complexity of vanilloid (capsaicin) receptors. For example, vanilloid receptors in the rat bind both resiniferatoxin and capsaicin in a positive cooperative fashion (Szallasi et al., 1993; Acs et al., 1995a) and display complex dissociation kinetics in that the off-rate depends on both the fractional receptor occupancy by resiniferatoxin at the time when the release was initiated and the pre-incubation period allowed for association (Szallasi and Blumberg, 1993b). Most recently, a [^{3}H]resiniferatoxin binding assay using rat dorsal root ganglia neurons cultured in vitro has been developed (Acs et al., 1995b) which allows a direct comparison of binding affinities of vanilloids to their functional potencies as determined in a Ca^{2+} uptake assay. An apparent dissociation between the binding affinity and functional potency of vanilloids was observed: resiniferanoids being less potent and capsaicinoids being more potent for induction of Ca^{2+} uptake than for binding, respectively (Acs et al., 1995b).

Ligand-gated ion channels have a multisubunit structure which permits a cooperative interaction among the subunits. For example, nicotinic acetylcholine receptors show positive cooperativity (Schiebler et al., 1977; Fels et al., 1982). Thus, it is hardly surprising that vanilloid receptors, believed to be ligand-gated cation channels (Bevan and Szolcsanyi, 1990; James et al., 1993), bind resiniferatoxin and capsaicin in a positive cooperative manner (Szallasi et al., 1993; Acs et al., 1995a). Also, it is easy to visualize a connection between binding cooperativity and unusual dissociation kinetics (Notides et al., 1981). The dissociation between binding affinity and functional potency of vanilloids (Acs et al., 1995b) is, however, puzzling.

As part of our efforts to define structural motifs which render resiniferatoxin an ultrapotent capsaicin analog, we have synthesized a series of vanilloids based on phorbol as well as 12-dehydrophorbol. We observed that these compounds not only inhibited [^{3}H]resiniferatoxin binding to rat spinal cord membranes but also affected (reduced) binding cooperativity, suggesting that this cooperative binding behaviour is a ligand-induced property rather than an inherent feature of vanilloid receptors. To explore this model, we have analyzed in detail the binding behaviour of phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPAHV), a paradigm of this novel class of vanilloid compounds.

As predicted by the modified Hill equation (Davis et al., 1977), non-radioactive resiniferatoxin affects [^{3}H]resiniferatoxin binding in a biphasic fashion, with an initial enhancement of binding followed by inhibition: the lower the fractional receptor occupancy by [^{3}H]resiniferatoxin, the greater the intial enhancement by the non-radioactive ligand. We have demonstrated this binding behaviour previously (Szallasi et al., 1993; Acs et al., 1995a) and confirm it here. By contrast, PPAHV has a monophasic effect on [^{3}H]resiniferatoxin binding: no detectable enhancement of binding precedes the inhibition. This finding suggests that PPAHV, unlike resiniferatoxin, binds to vanilloid receptors in rat spinal cord membranes in a non-cooperative manner. In keeping with this, the Scatchard plot of specific [^{3}H]resiniferatoxin binding determined in the presence of PPAHV is linear (the Hill coefficient is close to unity), indicating non-cooperative binding. This contrasts with the convex Scatchard plot determined under control conditions, indicative of positive cooperativity (a cooperativity index of 2). These findings suggest that not only does PPAHV bind to vanilloid receptors non-cooperatively but it also blocks conformational changes leading to positive cooperativity. This binding behaviour is surprising but not unprecedented; for example, estratrien-17β-ol affects the cooperativity of estrogen receptors in a similar fashion (Schwartz and Skafar, 1993). In parallel experiments, neither capsaicin nor capsazepine modified binding cooperativity. In addition, PPAHV turned the bi-phasic dissociation curve of resiniferatoxin into a monophasic curve, eliminating the second, slow off-rate phase. Taken together, these findings suggest that cooperative binding is a ligand-induced feature of vanilloid receptors, and that the second, slow-dissociation phase of resiniferatoxin binding is a result of the positive cooperativity. If this is the case, assay conditions may also influence binding cooperativity. In fact, recently we demonstrated that porcine dorsal horn membranes could bind resiniferatoxin in either a cooperative or a non-cooperative manner, depending on actual assay conditions

(Szallasi et al., 1994). Until the factors which modulate binding cooperativity are indentified, this binding parameter should not be used as a criterion to classify vanilloid receptors.

Resiniferatoxin is several thousand-fold more potent than capsaicin for binding to vanilloid receptors in the rat (Szallasi and Blumberg, 1990b; Szallasi, 1994 for reviews). This is in accord with the ultrapotency of resiniferatoxin as a capsaicin analog for most of the biological responses tested (Szallasi and Blumberg, 1990b; Blumberg et al., 1993 for reviews). Resiniferatoxin and capsaicin, however, show striking differences in relative potencies for different responses (Szallasi and Blumberg, 1990b, 1993a for reviews). For example, resiniferatoxin and capsaicin are equipotent for contracting the isolated rat urinary bladder altough resiniferatoxin is at least 1000-fold more potent than capsaicin for desensitizing the bladder to a subsequent capsaicin administration (Maggi et al., 1990). This also means that resiniferatoxin, unlike capsaicin, can desensitize the bladder without prior contractions (Maggi et al., 1990). Several hypotheses have been put forward to explain the diverse relative potencies of vanilloids, including pharmacokinetical differences (Maggi et al., 1990), a differential binding to plasma proteins (Blumberg et al., 1993), and vanilloid receptor heterogeneity (Szallasi and Blumberg, 1990b; Szallasi, 1994; Colquhoun et al., 1995). Whereas a combination of these factors may no doubt explain the contrasting relative potencies of resiniferatoxin and capsaicin for most biological end-points, the dissociation between the binding affinity of vanilloids to cultured dorsal root ganglia neurons and their potency to induce Ca^{2+} uptake by these cells is still puzzling (Acs et al., 1995b). It is possible that the Ca^{2+} uptake assay for reasons unknown to us minimizes the differences among the potency of vanilloids. However, we favor the alternative explanation, namely, that binding and Ca^{2+} uptake reflect distinct classes of receptors with different structure-activity relations. Based on the observations that resiniferanoids are less potent and capsaicinoids are more potent for the induction of Ca^{2+} uptake than for binding (Acs et al., 1995b), it might have been postulated that the receptor which mediates Ca^{2+} influx receives greater contribution from the homovanillyl constituent whereas the receptor detected in the present binding methodology receives a greater contribution from the diterpene moiety. This two-receptor model may be in accord with the observation that both resiniferatoxin and capsaicin bind to cultured dorsal root ganglia neurons in a positive cooperative fashion but induce Ca^{2+} uptake by them in a non-cooperative manner (Acs et al., 1995b). Interestingly, PPAHV, which structurally falls between the resiniferanoid and capsaicinoid classes of vanilloids, binds to cultured neurons and induces Ca^{2+} uptake by them with similar affinities. Thus, structure-activity relations for the above postulated receptors, if they exist, appear be more complex than thought previously.

The bottom line of this study is that positive cooperativity is a ligand-induced property of vanilloid receptors. It is hoped that a comparison of the spectrum of biological activity of ligands which bind to vanilloid receptors with different degrees of cooperativity will establish the role(s) for this binding behaviour. We suggest therefore that vanilloids be also evaluated for their effect on cooperativity and not only for their receptor binding affinity.

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