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High-Affinity Partial Agonists of the Vanilloid Receptor

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ABSTRACT

The vanilloid receptor VR1 is a polymodal nociceptor sensitive to capsaicin, protons, and heat. Because VR1 represents an attractive therapeutic target for conditions ranging from long-term pain to bladder hyperreflexia, we and other groups have sought to develop novel ligands with enhanced potencies and novel pharmacological properties. Here, we characterize two compounds, *N*-[2-(3,4-dimethylbenzyl)-3-(pivaloyloxy)propyl]-*N*′-[4-(methylsulfonylamino)benzyl]thiourea (JYL827) and *N*-(4-*tert*-butylbenzyl)-*N*′-[3-methoxy-4-(methylsulfonylamino)benzyl]thiourea (JYL1511), that function as partial agonists for rat VR1 heterologously expressed in Chinese hamster ovary cells. Both compounds showed substantially enhanced potency, inhibiting [^{3}H] resiniferatoxin binding with K_i values of 29.3 ± 7.6 and 50.4 ± 16.5 nM, respectively, compared with 1810 ± 270 nM for capsaicin. The compounds showed different extents of partial agonism, 6.8 ± 0.7% and 17.4 ± 0.6%, respectively, and the expected corresponding degrees of partial antagonism (93.9 ± 0.9 and 84.1 ± 3.2%, respectively). Their IC_{50} values for antagonism of $^{45}Ca^{2+}$ uptake in response to capsaicin were 67.3 ± 24.9 nM and 3.4 ± 0.5 nM, respectively. Protons, temperature, and protein kinase C all function as coactivators/modulators of rVR1. All enhanced the extent of partial agonism of JYL827 and JYL1511. Thus, at pH 5.5, for example, the extents of partial agonism increased to 54.9 ± 2.5% and to 90.7 ± 1.7%, respectively, relative to the response elicited by 300 nM capsaicin. The extents of partial antagonism decreased correspondingly. Compounds such as JYL827 and JYL1511 now permit exploration of the potential utility of partial agonists of rVR1 in animal models. Our results emphasize, moreover, the strong dependence of such partial agonists on other modulators of rVR1 and predict that their biological behavior will depend strongly on biological context.

The vanilloid receptor VR1 has attracted great attention, because of both its biological function and its therapeutic potential. VR1, also called vanilloid receptor type 1, a member of the transient receptor potential family of ion channels, is a modestly calcium-selective ion channel located in C-fiber and Aδ sensory neurons as well as in a growing number of other sites such as the central nervous system or the bladder (Szallasi, 2001). VR1 functions as an integrative transducer for a range of nociceptive signals, including heat, protons, endogenous ligands, such as lipoxygenase products or anandamide, and exogenous compounds such as capsaicin or resiniferatoxin (Julius and Basbaum, 2001). Activation of VR1 by capsaicin may be followed by subsequent loss of responsiveness, depending on dose, duration of application, and other conditions, and reflects a combination of overlapping mechanisms, such as dephosphorylation or calcium toxicity to the neurons (Szallasi and Blumberg, 1999). This desensitization/defunctionalization by capsaicin has been exploited to treat a variety of conditions in which C-fiber sensory neurons are involved, such as pain associated with arthritis, cystitis, human immunodeficiency virus, and diabetic neuropathy (Robbins, 2000).

Although capsaicin has made it possible to identify an exciting series of potential therapeutic applications, its utility has been limited by its somewhat modest potency, by the initial pain occasioned upon initial application, and by its metabolic lability (Szallasi, 2001). Attention has therefore been directed at the development and characterization of novel analogs of capsaicin. Although still in the early stages, much progress has been made.

Ligands have been identified with much greater potency for VR1 than that displayed by capsaicin. Resiniferatoxin (RTX), isolated from *Euphorbia resinifera*, is a natural product in which the alkyl C-region of capsaicin is replaced with a tricyclic diterpene structurally related to those found in the phorbol esters. RTX binds to rVR1 with an affinity 4 orders of

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ABBREVIATIONS: RTX, resiniferatoxin; JYL1511, *N*-(4-*tert*-butylbenzyl)-*N*′-[3-methoxy-4-(methylsulfonylamino)benzyl]thiourea; JYL827, *N*-[2-(3,4-dimethylbenzyl)-3-(pivaloyloxy)propyl]-*N*′-[4-(methylsulfonylamino)benzyl]thiourea; CHO, Chinese hamster ovary; CHO/rVR1 cell, Chinese hamster ovary cells transfected with rVR1; PBS, phosphate-buffered saline; DPBS, Dulbecco's modified PBS with Ca^{2+} and Mg^{2+}; MES, 2-[*N*-morpholino]ethanesulfonic acid; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; rVR1, cloned rat vanilloid receptor subtype-1.

magnitude stronger than that of capsaicin (Szallasi et al., 1999a). Incorporating elements of the postulated pharmacophoric groups provided by the diterpene moiety of RTX into synthetic capsaicin analogs, we have characterized compounds with binding affinities for rVR1 up to 280-fold stronger than that of capsaicin (Lee et al., 2001a).

Antagonists for VR1 have also been developed. Capsazepine, the most extensively characterized, is a competitive antagonist of capsaicin with affinity similar to that of capsaicin (Bevan et al., 1992). A problem has been its limited selectivity; it also blocks nicotinic cholinergic receptors, voltage dependent calcium channels, and purinergic receptors at concentrations comparable with those at which it is active on VR1 (Docherty et al., 1997; Liu and Simon, 1997; Wardle et al., 1997). 5-Iodo-4-hydroxy-3-methoxy RTX shows markedly enhanced potency, with an IC_{50} for rat VR1 expressed in *Xenopus laevis* oocytes of 3.9 nM (40-fold stronger than that of capsazepine in this system) (Wahl et al., 2001). *N*-(4-*tert*-butylbenzyl)-*N*′-[3-fluoro-4-(methylsulfonylamino)-benzyl]thiourea (compound 1) (Suh et al., 2002a), one of a series of synthetic capsaicin analogs with antagonistic activity, gives an IC_{50} for rat VR1 expressed in CHO cells of 9.2 nM (60-fold stronger than that of capsazepine in this system) (Wang et al., 2002).

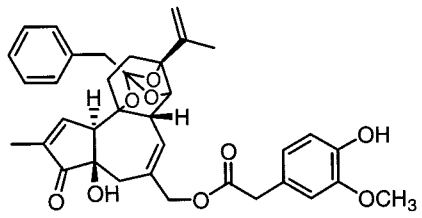
Metabolism can be restricted, with marked enhancement in activity upon oral administration, as exemplified by *N*-(4-(2-aminoethoxy)-3-methoxybenzyl)-*N*′-(4-*tert*-butylbenzyl)thiourea. This compound was 2-fold more potent than capsaicin for inducing Ca^{2+} influx in cultured cells but 640-fold more potent than capsaicin in the mouse tail-flick assay upon oral administration (Wrigglesworth et al., 1996).

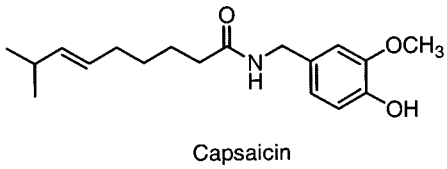
Finally, different endpoints of biological response to vanilloids have been shown to be separable, at least partially. Of particular interest is that compounds such as olvanil or RTX show reduced pungency relative to their ability to desensitize (Szallasi and Blumberg, 1989; Liu et al., 1997). Likewise, effects on inflammation can be dissociated from those on thermoregulation (Szallasi et al., 1999b).

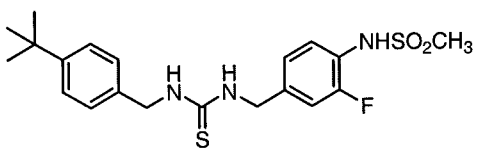
An ongoing effort of this group has been to exploit the postulated pharmacophoric groups of RTX, as well as other strategies, to generate capsaicin analogs with enhanced potency and novel properties (Lee et al., 1999, 2001a,b; 2002). We have identified multiple derivatives that show partial efficacy relative to capsaicin in their ability to induce calcium uptake in cells expressing rVR1. We characterize here in detail the activity of two compounds, selected because of somewhat different levels of partial efficacy. JYL827 illustrates a compound with quite limited agonism under standard conditions, which might cause it mistakenly to be treated as a full antagonist; JYL1511 illustrates a compound with a higher level of agonism. These compounds, the structures of which were initially described elsewhere (Suh et al., 2002a,b), are shown to function as partial agonists, and the extent of their partial agonism depends on the presence of coactivators such as protons, heat, or activation of protein kinase C.

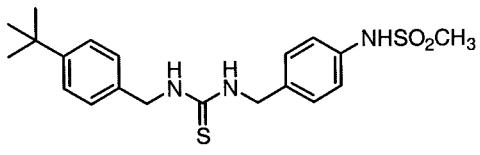
Materials and Methods

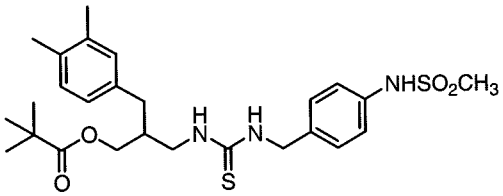
Materials. JYL1151 and JYL827 were synthesized as described elsewhere (Suh et al., 2002a,b). The structures of these compounds and of related structures are shown in Fig. 1. [^{3}H]RTX (37 Ci/mmol) was provided by PerkinElmer Life Sciences (Boston, MA). $^{45}Ca^{2+}$ was from ICN Biomedicals, Inc. (Irvine, CA). Nonradioactive RTX, capsaicin, and capsazepine were purchased from Alexis Corp (San Diego, CA).











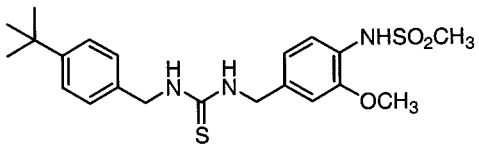


Fig. 1. Structures of JYL827 and JYL1511. Also shown for comparison are the structures of capsaicin and two full antagonists (Suh et al., 2002a; Wang et al., 2002). Compound 1, *N*-(4-*tert*-butylbenzyl)-*N*′-[3-fluoro-4-(methylsulfonylamino)benzyl]thiourea; compound 2, *N*-(4-*tert*-butylbenzyl)-*N*′-[4-(methylsulfonylamino-)benzyl]thiourea.

Preparation and Subculture of Cells Stably Expressing Rat VR1. Chinese hamster ovary (CHO) cells stably transfected with rat VR1 in a pTet off regulatory system were described previously (Szallasi et al., 1999a). In this system, expression of the rVR1 is repressed in the presence of tetracycline but is induced upon removal of the antibiotic. The cells were maintained in medium supplemented with tetracycline (1 μg/ml) (Szallasi et al., 1999a). Cells used for assays were grown in culture medium without tetracycline for 48 h before use. For radioligand binding experiments, cells were seeded in T75 cell culture flasks in the culture media with tetracycline (1 μg/ml) and G-418 (0.25 mg/ml). After 2 days, the culture medium was changed to medium without tetracycline and the cells were grown for an additional 48 h to induce rVR1 expression. The flasks were washed with PBS and the cells harvested in PBS containing 5 mM EDTA. The cells were pelleted by gentle centrifugation and stored at −20°C until assayed. For assay of $^{45}Ca^{2+}$ uptake, cells were seeded into 24-well plates in media with tetracycline (1 μg/ml) and G-418 (0.25 mg/ml). After 1 day, the culture medium was changed to medium without tetracycline and the cells were grown for an additional 48 h to induce VR1 expression. For calcium imaging, cells were grown on glass coverslips (25 mm).

Competition Binding Assay. Binding studies with [^{3}H]RTX were carried out as described previously with minor modifications (Wang et al., 2002). Binding assay mixtures were set up on ice and contained 80 pM [^{3}H]RTX, various concentrations of competing ligands, 0.25 mg/ml BSA (Cohn fraction V), and 5×10^4 to 10^5 CHO/rVR1 cells. The final volume was adjusted to 450 μl with DPBS containing Ca^{2+} and Mg^{2+} (Invitrogen, Gaithersburg, MD) and 0.25 mg/ml bovine serum albumin. Nonspecific binding was determined in the presence of 100 nM nonradioactive RTX. The binding reaction was initiated by transferring the assay mixtures to a 37°C water bath and was terminated after a 60-min incubation period by cooling the tubes on ice. Nonspecific binding was reduced by addition of 200 μg of bovine glycoprotein fraction VI (α-glycoprotein) (ICN, Costa Mesa, CA) to each tube. Membrane-bound RTX was separated from free RTX by pelleting the membranes in a model 12 Microfuge (15 min, maximal velocity; Beckman Coulter, Fullerton, CA); the tips of the tubes containing the pellets were cut off, and the radioactivity was determined by scintillation counting. Equilibrium binding parameters (K_i, B_{max}, and cooperativity) were determined by fitting the Hill equation to the measured values with the aid of the program Origin 6.0 (OriginLab Corp., Northampton, MA).

$^{45}Ca^{2+}$ Uptake. CHO/rVR1 cells were incubated for 5 min at 37°C or as indicated with 0.2 μCi/well $^{45}Ca^{2+}$ in the presence of serum-free DMEM, 0.25 mg/ml bovine serum albumin, and various concentrations of the different compounds. To determine the pH dependence of $^{45}Ca^{2+}$ uptake, cells were incubated for 5 min at 37°C with 0.2 μCi/well $^{45}Ca^{2+}$ in the presence of DPBS, supplemented with 0.25 mg/ml bovine serum albumin and various concentrations of the different compounds, adjusted to the indicated pH with 1 M MES (Sigma, St. Louis, MO). After incubation, cells were washed 3 times with DPBS and lysed in 400 μl/well of radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS) for 20 min. Aliquots of the solubilized cell extracts were counted in a liquid scintillation counter.

Imaging of Intracellular Calcium Levels $[Ca^{2+}]_i$. Cells grown on coverslips were loaded with Fura-2 AM (10 μM) (Molecular Probes, Eugene, OR) for 10 min at 37°C and an additional 50 min at room temperature (for CHO/rVR1 cells), washed, and then incubated at room temperature for at least an additional hour. Coverslips were placed in a chamber at room temperature. Images of Fura-2–loaded cells with the excitation wavelength alternating between 340 and 380 nM were captured using a Cohu 4915 low-light CCD camera on an InCyt Dual-Wavelength Fluorescence Imaging and Photometry System (Intracellular Imaging Inc., OH). The ratio of fluorescence intensity at the two wavelengths was calculated.

Results

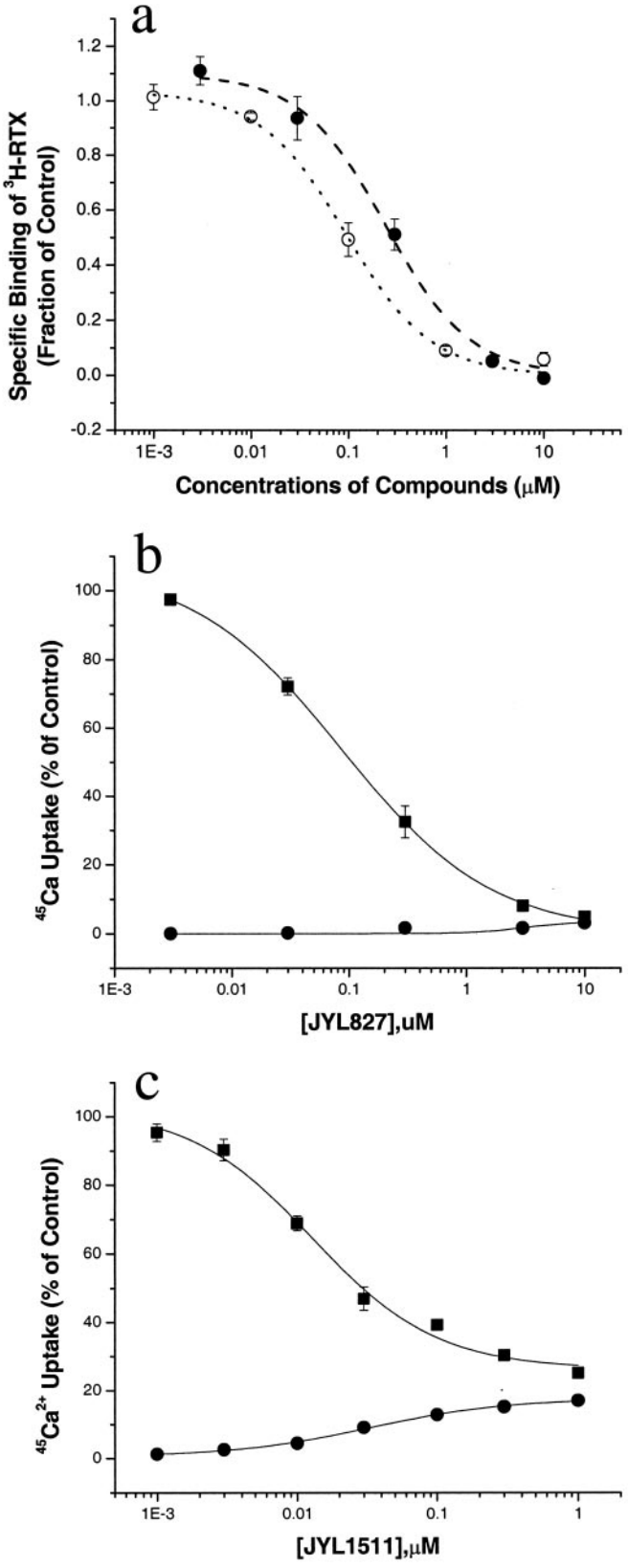
JYL827 and JYL1511 Potently Bind to Rat VR1. We have previously demonstrated that ligand binding to rat VR1 heterologously expressed in CHO cells or HEK293 cells closely resembles that characterized in rat dorsal root ganglion neurons (Szallasi et al., 1999a). We have therefore used the CHO/rVR1 system for determination of ligand structure activity relations. JYL827 and JYL1511 inhibited [^{3}H]RTX binding to rat VR1 with K_i values of 29.3 ± 7.6 nM (n = 6 experiments) and 50.4 ± 16.5 nM (n = 3 experiments), respectively (Fig. 2a). For comparison, the K_i of capsaicin under these conditions was 1810 ± 270 nM (J. Lee, J. Lee, M. Kang, M. Y. Shin, J. M. Kim, S. U. Kang, J. O. Lim, H. K. Choi, Y. G. Suh, H. G. Park, et al., submitted). The compounds were thus 60- and 35-fold more potent, respectively, than capsaicin for binding to rVR1.

JYL827 and JYL1511 Function as Partial Agonists/Partial Antagonists on Rat VR1. We evaluated agonism by the activation of $^{45}Ca^{2+}$ uptake by CHO/rVR1 cells upon incubation with compounds for 5 min. The levels of $^{45}Ca^{2+}$ uptake were compared with that induced by a saturating concentration of capsaicin (300 nM under these conditions). JYL827 and JYL1511 induced 6.8 ± 0.7% (n = 7 experiments) and 17.4 ± 0.6% (n = 6 experiments) of the level of $^{45}Ca^{2+}$ uptake induced by capsaicin. The EC_{50} values were 35.5 ± 4.2 nM (n = 4 experiments) and 32.4 ± 5.3 nM (n = 3 experiments), respectively (Fig. 2, b and c).

Although partial efficacy of these compounds might imply that they function as partial agonists on rat VR1, an alternative is that the compounds have difficulties crossing the plasma membrane to reach the ligand binding site of VR1, which is on the inner face of the membrane (Jung et al., 1999). We therefore examined the ability of the compounds to antagonize $^{45}Ca^{2+}$ uptake induced by 50 nM capsaicin when the compound and capsaicin were added simultaneously. We used the 50 nM concentration of capsaicin, approximately its EC_{50}, to minimize the rightward shift in antagonist dose response curves caused by competition with the capsaicin. Under these conditions, capsaicin induction of $^{45}Ca^{2+}$ uptake was antagonized both by JYL827 and by JYL1511 (Fig. 2, b and c). The levels of inhibition by the compounds of the $^{45}Ca^{2+}$ uptake induced by capsaicin were complementary to the levels of stimulation of $^{45}Ca^{2+}$ uptake by the compounds alone. Thus, JYL827 inhibited by 93.9 ± 0.9% (n = 7 experiments) and JYL1511 inhibited by 84.1 ± 3.2% (n = 6 experiments), giving total values for the percentage of agonism plus antagonism of 101 and 102%, respectively, as expected for a mechanism of partial agonism. The IC_{50} value of JYL827 for antagonism of $^{45}Ca^{2+}$ uptake induced by capsaicin was 67.3 ± 24.9 nM (n = 4 experiments); the IC_{50} value of JYL1511 was 3.4 ± 1.0 nM (n = 3 experiments). For JYL827, the EC_{50} and IC_{50} values show good agreement. For JYL1511, the EC_{50} value is higher (less potent), presumably reflecting the difficulties of quantitation when the extent of agonism is low. We conclude that JYL827 and JYL1511 are partial agonists/partial antagonists, representing different extents of partial agonism.

Activity of JYL827 and JYL1511 on CHO/RVR1 Cells as Evaluated by Calcium Imaging. Calcium imaging provides an alternative measure for VR1 responsiveness. In the CHO/rVR1 system, a maximally effective dose of JYL827 (3

μM) caused a barely measurable increase in $[Ca^{2+}]_i$, and JYL1511 (3 μM) caused an intermediate response, although clearly much less than that induced by 300 nM capsaicin (Fig. 3, a–c). These results are consistent with the results determined by $^{45}Ca^{2+}$ uptake.



VR1 Expression Level Governs the Efficacy of Partial Agonists. Receptor density is known to be a factor strongly influencing the efficacy of ligands in both tissue and recombinant systems, particularly in systems under conditions of spare receptors (Kenakin, 1997). In our CHO/rVR1 system, the expression of rVR1 is repressed by tetracycline and is induced upon removal of the antibiotic. By varying the concentration of tetracycline (0, 1, 5, 10 μg/ml) in the maintaining medium, we could obtain different levels of receptor expression in the CHO/rVR1 cells, which we measured by binding of [^{3}H]RTX under saturating conditions (Fig. 4a). With the increase in the expression of rVR1, JYL827 shifted from a full antagonist to a partial agonist (0 to 23 $\pm$ 0.56% of the level of $^{45}Ca^{2+}$ uptake induced by 300 nM capsaicin, n = 3 experiments) (Fig. 4b). For JYL1511, partial agonism increased from 17.4 $\pm$ 0.6 to 59 $\pm$ 1.2% (n = 3 experiments) relative to capsaicin (Fig. 4c). The maximum response of the full agonist capsaicin also increased as the level of expression of rat VR1 increased, but the magnitude was much less than that of the partial agonists (data not shown). We conclude that the level of VR1 expression has an important effect on whether a compound appears as an antagonist or partial agonist as well as on the degree of partial agonism.

Temperature Affects the Efficacy of Partial Agonists. Elevated temperature is a potent activator of VR1 and is a potentiator of the action of capsaicin on VR1 (Tominaga et al., 1998). We therefore examined the effect of temperature on the response of the CHO/rVR1 cells to JYL827 and JYL1511. In contrast to the full antagonists of capsaicin action that we described previously, neither JYL827 nor JYL1511 antagonized the $^{45}Ca^{2+}$ uptake induced by elevated temperature (Wang et al., 2002). Rather, increasing temperature enhanced the extent of partial agonism by the compounds (Fig. 5a). For JYL827, the extent of partial agonism increased from 6.8 $\pm$ 0.7% (n = 7 experiments) at 37°C to 21.3 $\pm$ 1.8% (n = 3 experiments) at 44°C. For JYL1511, which has more efficacy as an agonist, its extent of partial agonism increased from 17.4 $\pm$ 0.6% (n = 6 experiments) at 37°C to 37.7 $\pm$ 3.1% (n = 3 experiments) at 44°C. The extents of $^{45}Ca^{2+}$ uptake were expressed relative to those induced by capsaicin (300 nM) at the same temperature. Consistent with the expected behavior for partial agonists, increasing temperature reduced the extent of partial antagonism by the compounds (Fig. 5b). For JYL827, the extent of partial antagonism decreased from 93.9 $\pm$ 0.9% (n = 7 experiments) at 37°C to 74.8 $\pm$ 4.0% (n = 3 experiments) at 44°C. For JYL1511, which has less efficacy as an antagonist, the extent of partial antagonism decreased from 84.1 $\pm$ 3.2% (n = 6

Fig. 2. Activity of JYL827 and JYL1511 on CHO/rVR1 cells. A, inhibition of specific [^{3}H]RTX binding. (○), JYL827; (●), JYL1511. Points represent the mean $\pm$ S.E.M. of triplicate determinations in single experiments. All experiments repeated at least three times. b, activity of JYL827 for (●) induction of $^{45}Ca^{2+}$ uptake and for (■) inhibition of $^{45}Ca^{2+}$uptake induced by capsaicin. For evaluation of JYL827 as an agonist, $^{45}Ca^{2+}$ uptake in excess of that for the medium control was determined for the indicated concentrations of JYL827 and was expressed relative to that by a maximally effective dose of capsaicin (300 nM). For evaluation of JYL827 as an antagonist, $^{45}Ca^{2+}$ uptake in excess of that for the medium control was determined for the indicated concentrations of JYL827 in the presence of 50 nM capsaicin. Points represent mean values of four determinations in single, representative experiments; error bars indicate S.E.M. All experiments were repeated at least two additional times with similar results. c, activity of JYL1511 for induction (●) and for inhibition (■) of $^{45}Ca^{2+}$ uptake induced by capsaicin. Analysis was as described for JYL827.

experiments) at 37°C to 61.2 ± 3.5% (n = 3 experiments) at 44°C. The extents of $^{45}Ca^{2+}$ uptake were expressed relative to those induced by capsaicin (300 nM) at the same temperature. For capsaicin, the absolute levels of $^{45}Ca^{2+}$ uptake were similar at all three temperatures. We conclude that the extent of partial agonism and partial antagonism is not an intrinsic characteristic of the ligand but rather depends on other coregulators, in this case temperature.

Protons Enhanced the Efficacy of Partial Agonists. Protons represent another class of well-characterized agonists for VR1 and are potentiators of the action of capsaicin on VR1 (Tominaga et al., 1998). They are of particular interest because of the physiological role that acidosis is believed to play in inflammatory pain. We have previously shown that

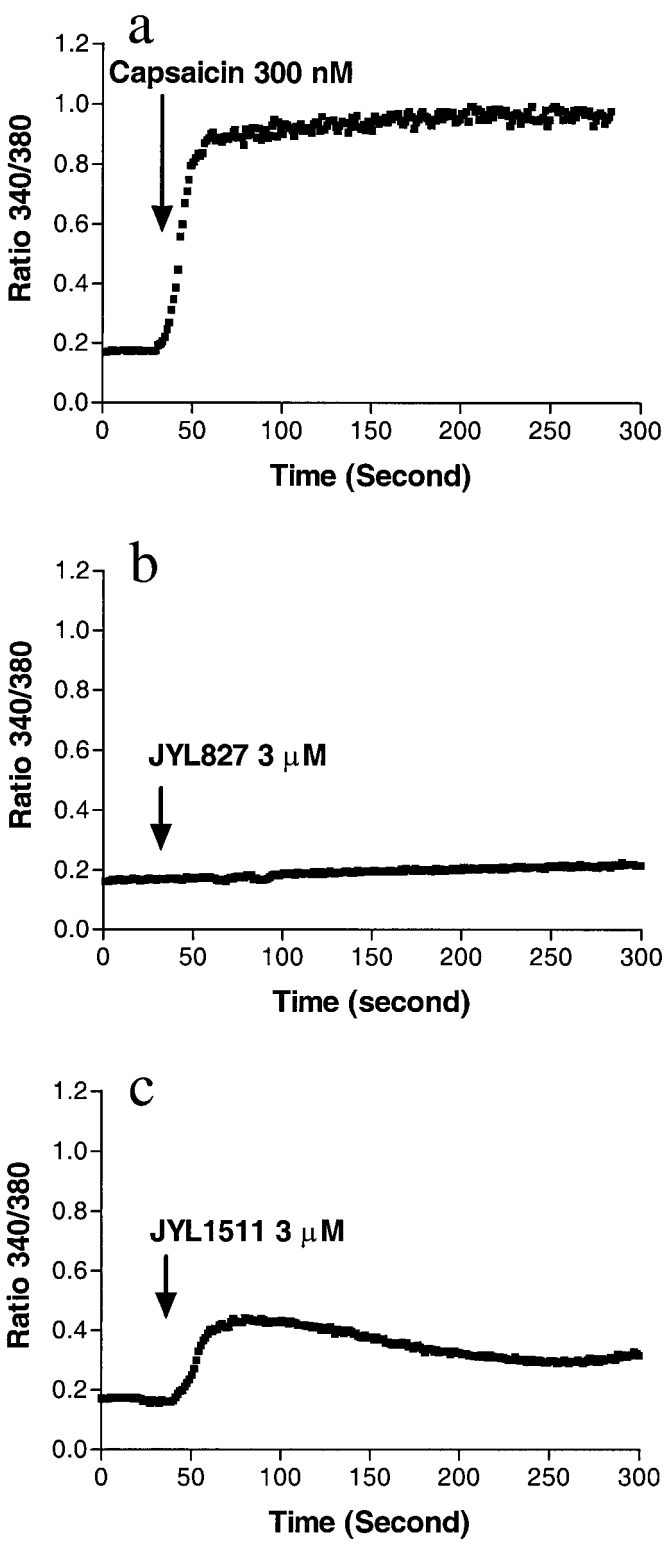


Fig. 3. Capsaicin and partial agonists evoked calcium mobilization in CHO/rVR1 cells as determined by calcium imaging. CHO/rVR1 cells were treated with capsaicin, JYL827, or JYL1511 (indicated by arrows). Points represent the averaged signal from a minimum of 16 cells imaged simultaneously. Each experiment was repeated at least an additional two times with similar results on independently cultured cells.

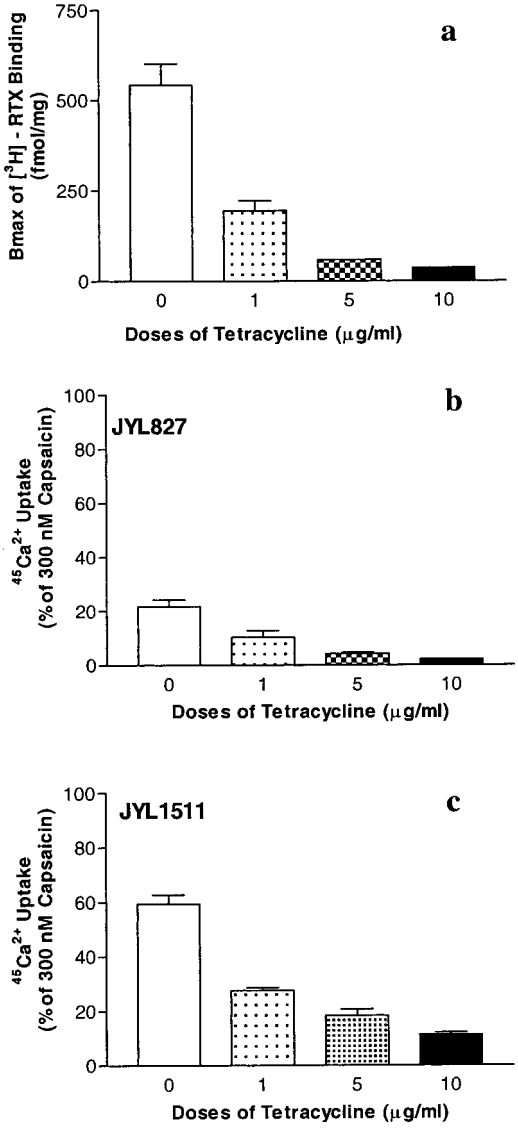


Fig. 4. $^{45}Ca^{2+}$ uptake evoked by partial agonists as a function of rat VR1 expression level. The CHO/rVR1 cells were cultured for 2 days in the presence of the indicated concentrations of tetracycline to give different levels of expression of rVR1. a, to compare levels of rVR1 expression, B_{max} values for [^{3}H]RTX binding (fmol/mg) were determined. The values were calculated from complete saturation binding curves in single, representative experiments. The experiment was repeated two additional times with similar results. b and c, $^{45}Ca^{2+}$ uptake induced by JYL827 (3 μM) and JYL1511 (3 μM). Levels of $^{45}Ca^{2+}$ uptake over baseline were expressed relative to that induced by 300 nM capsaicin. Points represent mean values of four determinations in single, representative experiments; error bars indicate S.E.M. All experiments were repeated at least two additional times with similar results.

different complete antagonists for capsaicin action on rat VR1 may fully or partially block the $^{45}Ca^{2+}$ uptake induced by a reduction in pH (Wang et al., 2002). We therefore examined $^{45}Ca^{2+}$ uptake in response to JYL827 and JYL1511 as the pH was reduced from pH 7.4 to 5.5 (Fig. 6). In contrast to the full antagonists of capsaicin action we described earlier, neither JYL827 nor JYL1511 antagonized the induction of $^{45}Ca^{2+}$ uptake by the lower pH. JYL827 stimulated $^{45}Ca^{2+}$ uptake, relative to that induced by capsaicin, by 6.8 ± 0.7% (n = 7 experiments) at pH 7.4 and by 54.9 ± 2.5% at pH 5.5 (n = 3 experiments). Conversely, the extent of partial antagonism of capsaicin induced $^{45}Ca^{2+}$ uptake decreased from 93.9 ± 0.9% (n = 7 experiments) at pH 7.4 to 42.9 ± 1.9% (n = 3 experiments) at pH 5.5.

JYL1511, as expected, showed a greater degree of agonism and less antagonism than did JYL827 at all pH values. The extent of partial agonism increased from 17.4 ± 0.6% (n = 6 experiments) at pH 7.4 to 90.7 ± 1.7% at pH 5.5 (n = 3 experiments), whereas the extent of partial antagonism decreased from 84.1 ± 3.2% (n = 6 experiments) at pH 7.4 to 8.0 ± 3.1% (n = 3 experiments) at pH 5.5. The extents of $^{45}Ca^{2+}$ uptake were expressed relative to those induced by capsaicin (300 nM) at the same pH. Once again, our results demonstrate that compounds cannot be regarded simply as antagonists or agonists for VR1; rather, their actions depend on the context in which VR1 is present.

The PKC Intracellular Signaling Pathway Can Potentiate Efficacy of Partial Agonists. It is well known that protein kinase C activation potentiates gating of the vanilloid receptor VR1 by capsaicin, protons, heat, and anandamide (Vellani et al., 2001; Crandall et al., 2002). Here, we examined the effect of the PKC activator PMA on the potency and efficacy of JYL827 and JYL1511 (Fig. 7). PMA (100 nM) increased the extent of partial agonism of JYL827 from 6.8 ± 0.6% (n = 7 experiments) to 17.1 ± 0.7% (n = 3 experiments) and that of JYL1511 from 17.4 ± 0.6% (n = 6 experiments) to 27.8 ± 1.0% (n = 3 experiments). Conversely, PMA decreased the extent of partial antagonism of JYL827 from 93.9 ± 0.9% (n = 7 experiments) to 82.8 ± 0.7% (n = 3 experiments) and that of JYL1511 from 84.1 ± 3.2% (n = 6 experiments) to 72.2 ± 1% (n = 3 experiments). We conclude that intracellular signaling pathways can also control the degree of partial agonism and antagonism.

Synergistic Effect of Coactivators on the Efficacy of Partial Agonists. We have described above that individual coactivators (protons, temperature, and PKC) can increase the extent of partial agonism and attenuate the extent of partial antagonism. Here, we examined the combined effect

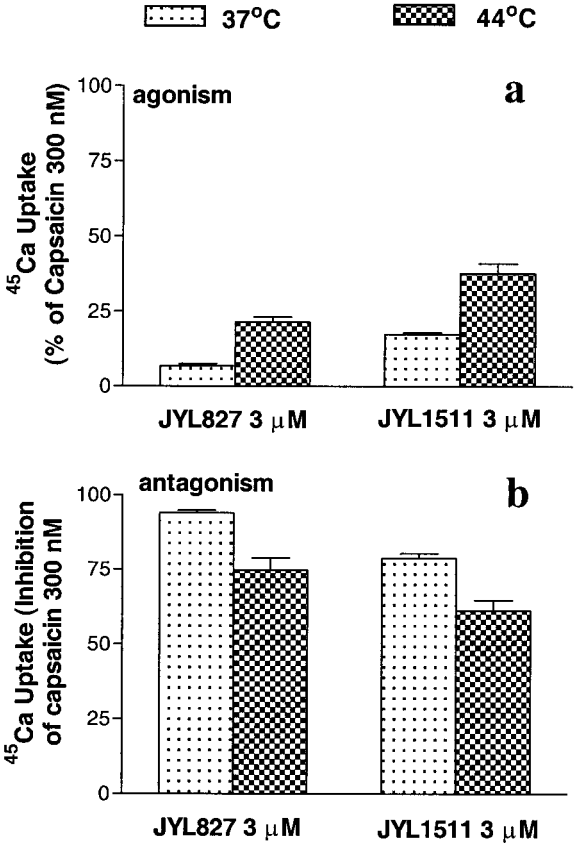


Fig. 5. Induction of $^{45}Ca^{2+}$ uptake in CHO/rVR1 cells by JYL827 and JYL1511 as a function of temperature. a, $^{45}Ca^{2+}$ uptake induced by JYL827 and JYL1511. Data are expressed as percentage of response induced by 300 nM capsaicin after subtraction of the baseline for medium alone. b, $^{45}Ca^{2+}$ uptake induced by JYL827 and JYL1511. Data are expressed as percentage of inhibition of the response induced by 300 nM capsaicin after subtraction of the baseline for medium alone. All values represent mean values of four determinations in three individual, representative experiments; error bars indicate S.E.M.

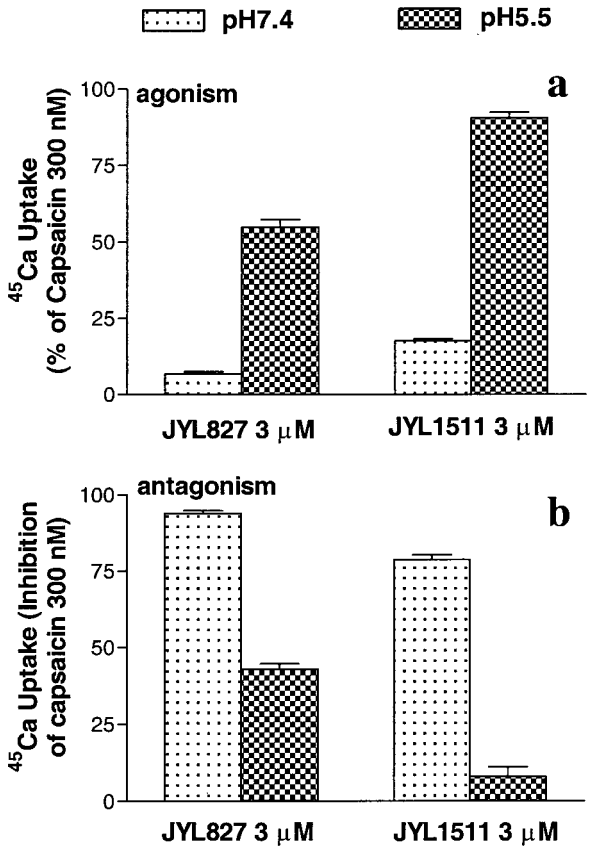


Fig. 6. $^{45}Ca^{2+}$ uptake of CHO/rVR1 cells evoked by partial agonists as a function of pH. a, $^{45}Ca^{2+}$ uptake induced by JYL827 and JYL1511 as a function of pH. Levels of $^{45}Ca^{2+}$ uptake over baseline were expressed relative to that induced by 300 nM capsaicin at the same pH. b, $^{45}Ca^{2+}$ uptake induced by JYL827 and JYL1511 as a function of pH. Data are expressed as percentage of inhibition of the response induced by 300 nM capsaicin after subtraction of the baseline for medium alone. All values represent mean values of four determinations in three individual, representative experiments; error bars indicate S.E.M.

of these three coactivators on the partial agonism/antagonism of JYL827 and JYL1511 (Fig. 8). The three coactivators functioned together to increase the extent of partial agonism of JYL 827 from 6.8 ± 0.7% (n = 7 experiments) to 89.4 ± 2.8% (n = 3 experiments) and that of JYL1511 from 17.4 ± 0.6% (n = 6 experiments) to 98.1 ± 2.6% (n = 3 experiments). Conversely, the combination of coactivators reduced the extent of antagonism of JYL827 from 93.9 ± 0.3% (n = 7 experiments) to 6.3 ± 3.2% (n = 3 experiments) and that of JYL1511 from 84.1 ± 3.2% (n = 6 experiments) to no antagonism. We conclude that protons, high temperature, and PKCs together further enhance the extent of partial agonism and, in the case of JYL827, convert a compound from virtually a complete antagonist (at 22°C, data not shown) to virtually a complete agonist.

Discussion

Partial agonists for VR1 have received relative little attention. Walpole and Wrigglesworth (1993) have described reduced efficacy (20–40%) for *meta*-chloro/fluoro, *p*-hydroxyphenyl derivatives of capsaicin. Likewise, 2-iodo-4-hydroxy-5-methoxy-RTX was reported to display 50 ± 13% of the efficacy of capsaicin (McDonnell et al., 2002), in contrast to 4-hydroxy-5-iodo-3-methoxy RTX, which was a complete antagonist (Wahl et al., 2001). In neither case was it shown that the fractional efficacy arose from partial agonism. This concern is not simply theoretical, because we have observed other compounds that show reduced efficacy without antagonism, presumably reflecting the complexities of the $^{45}Ca^{2+}$ assay (J. Lee, J. Lee, M. Kang, M. Y. Shin, J. M. Kim, S. U. Kang, J. O. Lim, H. K. Choi, Y. G. Suh, H. G. Park, et al., submitted). Finally, anandamide has been described as having partial efficacy for rat (Zygmunt et al., 1999) but not human VR1 (Smart et al., 2000). In the case of anandamide, transporter-dependent uptake and metabolism contribute to its apparent partial efficacy (De Petrocellis et al., 2001).

For JYL827 and JYL1511, we have demonstrated here that their fractional efficacy reflects their functioning as partial agonists with a corresponding degree of partial antagonism. The compounds further provide insights into structure activity relationships. The structure of JYL1511 demonstrates that the 4-methylsulfonylamino group on the A-region by itself does not assure antagonism. Rather, the complete antagonism of compound 2 (*N*-(4-*tert*-butylbenzyl)-*N*′-[4-(methylsulfonylamino)benzyl]thiourea) (Suh et al., 2002a; Wang et al., 2002) is converted into the partial agonism/antagonism of

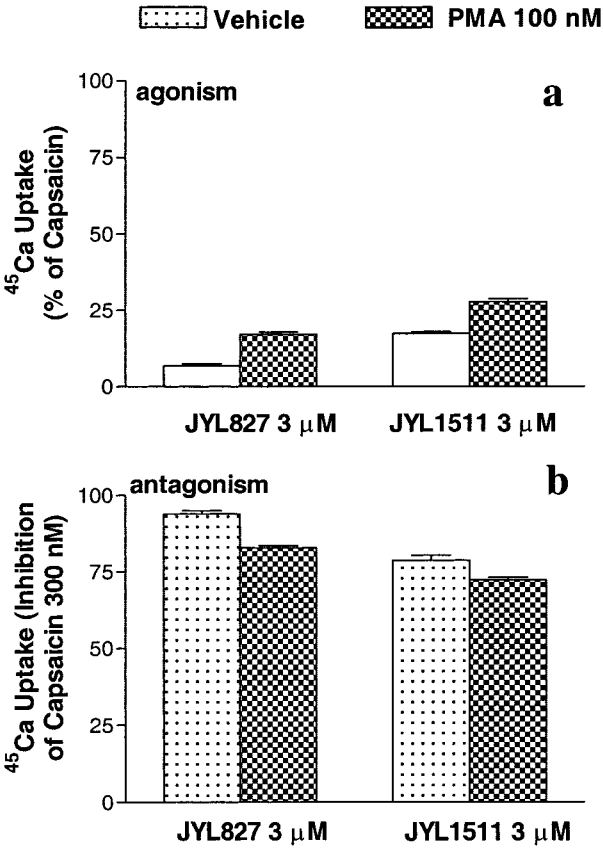


Fig. 7. $^{45}Ca^{2+}$ uptake of CHO/rVR1 cells evoked by partial agonists in the absence or presence of PMA. a, $^{45}Ca^{2+}$ uptake induced by JYL827 and JYL1511 as a function of PMA treatment. Levels of $^{45}Ca^{2+}$ uptake over baseline were expressed relative to that induced by 300 nM capsaicin at the same concentration of PMA. b, $^{45}Ca^{2+}$ uptake induced by JYL827 and JYL1511 as a function of PMA treatment. Data are expressed as percentage of inhibition of the response induced by 300 nM capsaicin after subtraction of the baseline for medium alone. All values represent mean values of four determinations in three individual, representative experiments; error bars indicate S.E.M.

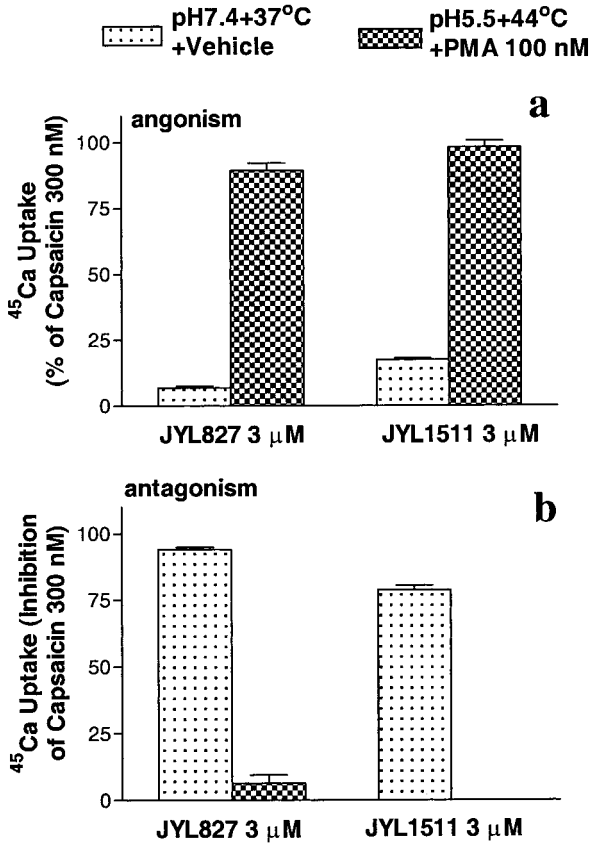


Fig. 8. Effect of the combination of protons, temperature, and PMA on the extent of partial agonism/antagonism. a, $^{45}Ca^{2+}$ uptake induced by JYL827 and JYL1511 as a function of protons, temperature, and PMA. Levels of $^{45}Ca^{2+}$ uptake over baseline were expressed relative to that induced by 300 nM capsaicin under the same conditions. b, $^{45}Ca^{2+}$ uptake induced by JYL827 and JYL1511 as a function of protons, temperature, and PMA. Data are expressed as percentage of inhibition of the response induced by 300 nM capsaicin under the same conditions after subtraction of the baseline for medium alone. All values represent mean values of four determinations in three individual, representative experiments; error bars indicate S.E.M.

JYL1511 by the presence of the additional 3-methoxy group. Furthermore, although the A-region makes a major contribution to the extent of agonism/antagonism, it is clear that the C-region also contributes. Thus, replacement of the *N*-[2-(3,4-dimethylbenzyl)-3-(pivaloyloxy)propyl] moiety in the partial agonist JYL827 with the *N*-(4-*tert*-butylbenzyl) moiety (compound 2) (Suh et al., 2002a; Wang et al., 2002) converted it into a complete antagonist.

A noteworthy feature of the partial agonists described here, differentiating them from the full antagonists for capsaicin action that we described previously (Wang et al., 2002), was that the partial agonists inhibited only the response to capsaicin but not that to pH or temperature. These latter stimuli potentiated the response to the partial agonists. Whether other compounds might show a different pattern of response remains to be determined.

JYL827 and JYL1511 show that it is possible to attain variable degrees of partial agonism. Thus, under our usual assay conditions JYL827 showed less agonism than did JYL1511. Moreover, for both JYL827 and JYL1511, the degree of agonism was a function of the context in which VR1 was found. Consistent with the results in many systems (Kenakin, 1997), the level of receptor expression was an important determinant of the degree of partial agonism. In addition, temperature, pH, and protein kinase C, three well characterized coactivators of rVR1, all served to enhance the extent of agonism. Although the basis for this synergistic enhancement is not yet understood, it cannot be explained simply by a change in ligand binding affinity, because responses at maximally stimulatory concentrations were determined.

Our findings have implications for screening of VR1 antagonists. We have shown how JYL827 could appear as virtually a complete antagonist at 22°C or as an agonist of good efficacy (89.4% efficacy) at pH 5.5, 44°C in the presence of PMA. The situation was similar as a function of VR1 expression level, where JYL827 shifted from no agonism at a low level of VR1 expression to 23% efficacy at a higher level of expression. Therefore, for consistent results in evaluation of antagonists, assay conditions need to be carefully controlled.

Our findings have potential therapeutic implications as well. It has been suggested that a slow rate of uptake of olvanil into the cell may be responsible for the reduced pungency of this compound (Liu et al., 1997), thereby giving olvanil a more favorable therapeutic index. In a similar fashion, it is possible that partial agonists, by providing a more limited influx of calcium, may differentially affect response and desensitization/defunctionalization of sensory neurons.

Moreover, we have shown here that the behavior of partial agonists is dependent on the cellular context in which VR1 is found. An important implication of these results is that partial agonists may behave differently on different subsets of VR1-containing cells, whether they are distinguished by cell type or by environment, and therefore that different partial agonists may be optimal for different conditions. An underlying conceptual problem with VR1 therapeutics is how to achieve a local effect from systemic administration. The modulated behavior of partial agonists may provide one approach. For example, inflammation may be associated with both a locally lowered pH as well as the release of inflammatory mediators such as bradykinin, which can lead to PKC activation. A compound such as JYL827 should preferentially activate VR1 in this environment and might thereby give local desensitization/defunctionalization. Partial agonists such as JYL827 or JYL1511, together with those that may be developed through other synthetic programs, may permit such concepts to be further evaluated.

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