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High Affinity Antagonists of the Vanilloid Receptor

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ABSTRACT

The vanilloid receptor VR1 has attracted great interest as a sensory transducer for capsaicin, protons, and heat, and as a therapeutic target. Here we characterize two novel VR1 antagonists, KJM429 [*N*-(4-*tert*-butylbenzyl)-*N*′-[4-(methylsulfonylamino)benzyl]thiourea] and JYL1421 [*N*-(4-*tert*-butylbenzyl)-*N*′-[3-fluoro-4-(methylsulfonylamino)benzyl]-thiourea], with enhanced activity compared with capsazepine on rat VR1 expressed in Chinese hamster ovary (CHO) cells. JYL1421, the more potent of the two novel antagonists, inhibited [^{3}H]resiniferatoxin binding to rVR1 with an affinity of 53.5 ± 6.5 nM and antagonized capsaicin-induced calcium uptake with an EC_{50} of 9.2 ± 1.6 nM, reflecting 25- and 60-fold greater potencies than capsazepine. Both JYL1421 and KJM429 antagonized RTX as well as capsaicin and their mechanism was competitive. The responses to JYL1421 and KJM429 differed for calcium uptake by rVR1 induced by heat or pH. JYL1421 antagonized the response to both pH 6.0 and 5.5, whereas KJM429 antagonized at pH 6.0 but was an agonist at lower pH (<5.5). For heat, JYL1421 fully antagonized and KJM429 partially antagonized. Capsazepine showed only weak antagonism for both pH and heat. Responses of rVR1 to different activators could thus be differentially affected by different ligands. In cultured dorsal root ganglion neurons, JYL1421 and KJM429 likewise behaved as antagonists for capsaicin, confirming that the antagonism is not limited to heterologous expression systems. Finally, JYL1421 and KJM429 had little or no effect on ATP-induced calcium uptake in CHO cells lacking rVR1, unlike capsazepine. We conclude that JYL1421 is a competitive antagonist of rVR1, blocking response to all three of the agonists (capsaicin, heat, and protons) with enhanced potency relative to capsazepine.

A vanilloid receptor (VR1) that is activated by capsaicin, low pH, and temperatures higher than 42°C has been cloned from rat dorsal root ganglia (Caterina et al., 1997; Tominaga et al., 1998). It is a nonselective cation channel, with high permeability for divalent cations, expressed on unmyelinated pain-sensing nerve fibers (C-fibers) and small Aδ fibers in the dorsal root, trigeminal, and nodose ganglia. Initially, activation of VR1 by pungent agonists such as capsaicin leads to excitation of primary sensory neurons gating nociceptive inputs to the central nervous system. Subsequently, these fibers may become desensitized/defunctionalized, and this desensitization forms a basis for the therapeutic use of VR1 agonists (Szallasi and Blumberg, 1999). Potential therapeutic applications include detrusor hyperreflexia, postherpetic neuralgia, diabetic neuropathy, cluster headache, osteoarthritis, and pruritus (Rains and Bryson, 1995; Kim and Chancellor, 2000).

The exciting potential therapeutic applications for vanilloids have motivated efforts to identify or design novel derivatives with improved properties (Walpole et al., 1993a,b,c; Wrigglesworth et al., 1996). An important advance was the identification of resiniferatoxin (RTX), a diterpene related to the phorbol esters, as an ultrapotent capsaicin analog (Szallasi and Blumberg, 1989). RTX demonstrated that orders of magnitude of additional affinity for VR1 could be captured through appropriate chemistry. Furthermore, because RTX was much more potent than capsaicin for desensitization, whereas it was only modestly more potent for inducing acute pain, as determined in the eye wiping assay, the behavior of RTX strongly suggested that these different biological endpoints could be dissociated, at least in part. This was important, because desensitization is a therapeutic goal, whereas the induction of acute pain is the limiting

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ABBREVIATIONS: RTX, resiniferatoxin; HEK, human embryonic kidney; CHO, Chinese hamster ovary; rVR1, cloned rat vanilloid receptor subtype-1; CHO/rVR1 cell, Chinese Hamster Ovary cells transfected with cloned rat vanilloid receptor subtype-1; DRG, dorsal root ganglion; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; JYL1421, *N*-(4-*tert*-butylbenzyl)-N′-[3-fluoro-4-(methylsulfonylamino)benzyl]thiourea; KJM429, *N*-(4-*tert*-butylbenzyl)-N′-[4-(methylsulfonylamino)benzyl]thiourea; DPBS, Dulbecco's phosphate-buffered saline; PBS, phosphate-buffered saline; CPZ, capsazepine; MES, 2-[*N*-Morpholino]ethanesulfonic acid.

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toxicity for capsaicin. Because of its favorable therapeutic index, RTX is currently in clinical trials (Kim and Chancellor, 2000).

Desensitization of C-fiber sensory neurons represents one attractive therapeutic strategy. A complementary strategy is through pharmacological antagonists for VR1. Because of the long duration of desensitization after treatment with VR1 agonists, antagonists may be of particular utility when short-term blockade of VR1 is desired. So far, only a single antagonist of VR1, capsazepine, has been studied extensively (Bevan et al., 1992). Unfortunately, capsazepine has only modest potency and is somewhat nonspecific, also antagonizing voltage sensitive calcium channels and the nicotinic cholinergic receptor (Docherty et al., 1997; Liu and Simon, 1997; Wardle et al., 1997).

The efforts of several groups have thus been directed at the development of novel antagonists. Exploiting the high potency of RTX, Wahl et al. (2001) have described 5-iodo-RTX as a potent VR1 antagonist with a K_d of 4.3 nM for binding to rat VR1 heterologously expressed in human embryonic kidney (HEK) 293 cells and with an IC_{50} of 3.9 nM for inhibiting the capsaicin-induced membrane current in *X. laevis* oocytes expressing rat VR1. Compared with capsazepine in this system, 5-iodo-RTX was thus 40-fold more potent. Although the binding of 5-iodo-RTX was reversible, its antagonism was not competitive with capsaicin, suggesting that further study of its mechanism is still needed. In vivo, intrathecal administration of 5-iodo-RTX to mice blocked the acute pain response to injection of capsaicin. For human VR1, 5-iodo-RTX was reported to antagonize with an ID_{50} of 27 nM (McDonnell et al., 2002).

In a complementary approach, channel blockers of VR1 were prepared through combinatorial chemistry of *N*-alkyl glycine trimers (Garcia-Martinez et al., 2002). Two derivatives with IC_{50} values of 0.7 and 2.6 μM were described. These compounds were noncompetitive with capsaicin, as expected, and showed selectivity for VR1 relative to several other channels.

In our ongoing effort to design improved vanilloids, we have identified motifs conferring significantly enhanced potency for rVR1 agonists (Lee et al., 1999, 2001a, 2002). Based on these structures, we have designed a series of derivatives that function as rVR1 antagonists (Lee et al., 2001b; 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.-K. Park, et al., manuscript in preparation). In the present study, we have characterized in detail, using CHO cells heterologously expressing rVR1, two of these antagonists, KJM429 and JYL1421. JYL1421, in particular, is 60-fold more potent than capsazepine as a capsaicin antagonist and blocks the action of all three VR1 agonists—capsaicin, heat, and protons.

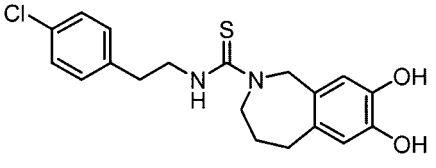
Materials and Methods

Materials. The antagonists JYL1421 [N-(4-*tert*-butylbenzyl)-*N*′-[3-fluoro-4-(methylsulfonylamino)benzyl]thiourea] and KJM429 [N-(4-*tert*-butylbenzyl)-*N*′-[4-(methylsulfonylamino)benzyl]thiourea] were synthesized as described below. The structures of these two compounds and of capsazepine are shown in Fig. 1. [^{3}H]RTX (37 Ci/mmol) was provided by PerkinElmer Life Science (Boston, MA). ^{45}Ca was from ICN Biomedicals, Inc. (Irvine, CA). Nonradioactive RTX, capsaicin, and capsazepine were purchased from Alexis Corp (San Diego, CA).

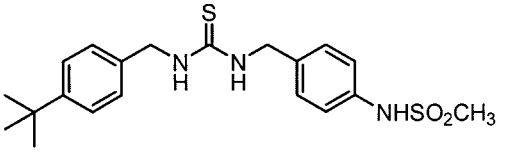
Synthesis of KJM429. A mixture of 4-aminobenzylamine (10 g, 82 mmol) and di-*tert*-butyl dicarbonate (20 g, 90 mmol) in THF (100 ml) was stirred for 2 h at room temperature and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with EtOAc/hexanes (2:3) as eluant to afford *tert*-butyl *N*-(4-aminobenzyl)carbamate as a white solid (17.5 g, 96%): m.p. = 75°C; ^{1}H NMR ($CDCl_3$) δ 7.06 (d, 2 H, J = 8.3 Hz, H-2,6), 6.63 (dt, 2 H, J = 2.7, 8.3 Hz, H-3,5), 4.73 (bs, 1 H, NHBoc), 4.18 (d, 2 H, J = 5.6 Hz, CH_2NH), 3.60 (bs, 2 H, NH_2), 1.45 (s, 9 H, $C(CH_3)_3$).

A cooled solution of the above amine (1.11 g, 5 mmol) at 0°C in pyridine (10 ml) was treated with methanesulfonyl chloride (0.464 ml, 6 mmol) and stirred for 16 h at room temperature. The reaction mixture was cooled in an ice-bath, neutralized carefully with 1 N hydrochloric acid solution, diluted with water, and extracted several times with dichloromethane. The combined organic layers were washed with water and brine, dried over magnesium sulfate, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with EtOAc/hexanes (1:1) as eluant to afford *tert*-butyl *N*-[(4-methylsulfonylamino)benzyl]carbamate as a white solid (1.38 g, 92%): m.p. = 162°C; ^{1}H NMR ($CDCl_3$) δ 7.28 (d, 2 H, J = 8.3 Hz, H-2,6), 7.18 (dd, 2 H, J = 2.2, 6.3 Hz, H-3,5), 6.76 (s, 1 H, $NHSO_2$), 4.88 (bs, 1 H, NHBoc), 4.28 (d, 2 H, J = 5.6 Hz, CH_2NH), 2.99 (s, 3 H, SO_2CH_3), 1.46 (s, 9 H, $C(CH_3)_3$).

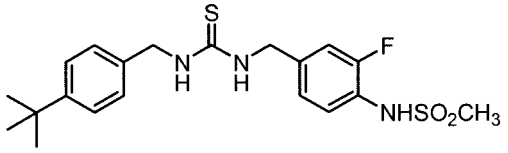
A cooled solution of the above compound (0.6 g, 2 mmol) in dichloromethane (10 ml) in an ice-bath was treated with trifluoroacetic acid (2.5 ml), which was added slowly, and the mixture was stirred for 1.5 h at 0°C. The mixture was concentrated in vacuo carefully to afford 4-(methylsulfonylamino)benzyl amine salt as a white solid in



Capsazepine



KJM429



JYL1421

Fig. 1. Structures of KJM429, JYL1421, and capsazepine.

a quantitative yield. The salt was washed with diethyl ether and used for the next step without further purification: ^{1}H NMR (DMSO-d_6) δ 9.87 (s, 1 H, $NHSO_2$), 8.14 (bs, 3 H, NH_3), 7.40 (d, 2 H, J = 8.5 Hz, H-2,6), 7.22 (d, 2 H, J = 8.5 Hz, H-3,5), 3.97 (s, 2 H, CH_2), 2.99 (s, 3 H, SO_2CH_3).

A solution of above amine salt (2 mmol) in dimethylformamide (4 ml) was treated with triethylamine (0.28 ml, 2 mmol) and stirred for 1 h at room temperature. To the mixture was added 4-*t*-butylbenzyl isothiocyanate (0.41 g, 2 mmol). After being stirred for 24 h at room temperature, the mixture was diluted with water and extracted several times with ethyl acetate. The combined organic layers were washed with water and brine, dried over magnesium sulfate, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with EtOAc/hexanes (2:3) as eluant to afford KJM429 as a white solid (0.73 g, 90%): m.p. = 83°C; ^{1}H NMR ($CDCl_3$) δ 7.37 (d, 2 H, J = 8.3 Hz), 7.18–7.25 (m, 4 H), 7.15 (d, 2 H, J = 8.3 Hz), 6.53 (s, 1 H, $NHSO_2$), 6.12 (bs, 1 H, NHCS), 5.96 (bs, 1 H, NHCS), 4.66 (d, 2 H, J = 5.4 Hz, $NHCH_2$), 4.57 (d, 2 H, J = 5.2 Hz, $NHCH_2$), 3.00 (s, 3 H, SO_2CH_3), 1.31 (s, 9 H, $C(CH_3)_3$); MS m/z 405 (M^+); Anal. Calcd for $C_{20}H_{27}N_3O_2S_2$: C, 59.23; H, 6.71; N, 10.36; S, 15.81. Found: C, 59.44; H, 6.73; N, 10.32; S, 15.79.

Synthesis of JYL1421. A cooled solution of 2-fluoro-4-iodoaniline (5 g, 21.1 mmol) in pyridine (30 ml) at 0°C was drop-wise treated with methanesulfonyl chloride (2.45 ml, 31.6 mmol). After being stirred for 3 h at room temperature, the mixture was diluted with water and extracted with ethyl acetate several times. The combined organic layers were washed with water and brine, dried over sodium sulfate, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with EtOAc/hexanes (1:2) as eluant to afford *N*-(2-fluoro-4-iodophenyl)methanesulfoamide as a white solid (5.32 g, 80%): m.p. = 118°C; ^{1}H NMR ($CDCl_3$) δ 7.50 (d, 2 H, J = 8.0 Hz), 7.33 (t, 1 H, J = 8.0 Hz), 6.54 (bs, 1 H, NH), 3.04 (s, 3 H, SO_2CH_3).

A mixture of the above iodide (5.32 g, 17 mmol), zinc cyanide (1.2 g, 10.2 mmol), and tetrakis(triphenylphosphine) palladium (0.976 g, 0.85 mmol) in dimethylformamide (15 ml) was heated at 80°C for 8 h. The mixture was diluted with water and extracted with ethyl acetate several times. The combined organic layers were washed with water and brine, dried over sodium sulfate, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with EtOAc/hexanes (1:2) as eluant to afford *N*-(2-fluoro-4-cyanophenyl)methanesulfoamide as a white solid (3.21 g, 87%): m.p. = 203°C; ^{1}H NMR ($CDCl_3$) δ 7.73 (t, 1 H, J = 8.3 Hz), 7.47 (m, 2 H), 7.00 (bs, 1 H, NH), 3.14 (s, 3 H, SO_2CH_3).

A stirred suspension of the above nitrile (2.142 g, 10 mmol), 5% palladium on carbon (0.2 g) and several drops of concentrated hydrochloric acid in MeOH (50 ml) was hydrogenated under a balloon of hydrogen for 16 h. The reaction mixture was filtered and the filtrate was concentrated in vacuo to afford 3-fluoro-4-(methylsulfonylamino)benzyl amine salt as a white solid, which was used for the next coupling step without further purification: m.p. = 256°C (decompose); ^{1}H NMR (CD_3OD) δ 7.56 (t, 1 H, J = 8.3 Hz), 7.28 (dd, 2 H), 4.07 (s, 2 H, CH_2), 2.98 (s, 3 H, SO_2CH_3).

A solution of above amine salt (2 mmol) and triethylamine (0.28 ml, 2 mmol) in dimethylformamide (2 ml) was stirred at room temperature for 30 min and treated with 4-*t*-butylbenzyl isothiocyanate (0.41 g, 2 mmol). After being stirred at room temperature for 20 h, the mixture was diluted with water and extracted with ethyl acetate several times. The combined organic layers were washed with water and brine, dried over magnesium sulfate, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with EtOAc/hexanes (1:1) as eluant to afford JYL-1421 as a white solid (0.762 g, 90%): m.p. = 59°C; ^{1}H NMR ($CDCl_3$) δ 7.47 (t, 1 H, J = 8.3 Hz), 7.38 (d, 2 H, J = 8.3 Hz), 7.23 (d, 2 H, J = 8.3 Hz), 7.02 (m, 2 H), 6.54 (s, 1 H, $NHSO_2$), 6.29 (bs, 1 H, NHCS), 6.01 (bs, 1 H, NHCS), 4.72 (d, 2 H, J = 5.6 Hz, $NHCH_2$), 4.54 (d, 2 H, J = 5.4 Hz, $NHCH_2$), 3.00 (s, 3 H, SO_2CH_3), 1.31 (s, 9 H, $C(CH_3)_3$).; MS m/z 423

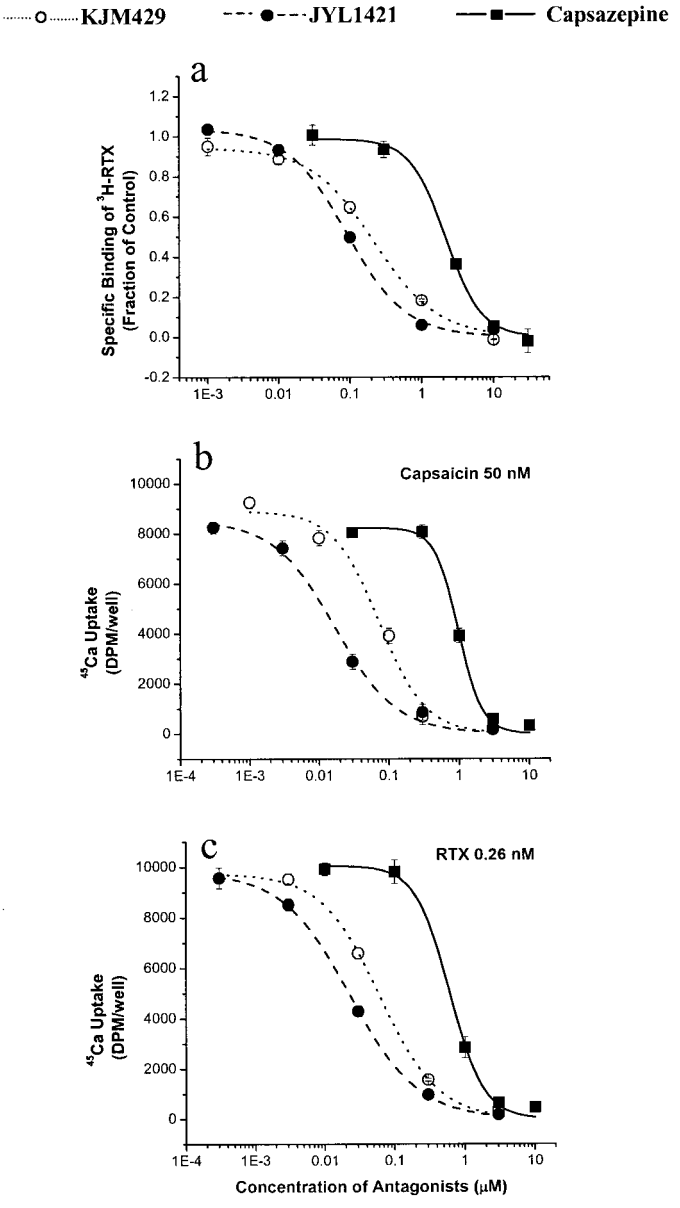


Fig. 2. Comparison of antagonists for inhibition of specific [^{3}H]RTX binding and of ^{45}Ca uptake induced by capsaicin and RTX. Ca^{2+} uptake was induced by 50 nM capsaicin or 0.26 nM RTX in the presence of the indicated concentrations of the antagonists. Points represent mean values of quadruplicate determinations in single, representative experiments; error bars indicate S.E.M. All experiments were repeated at least two additional times with similar results.

(M^+); Anal. Calcd for $C_{20}H_{26}FN_3O_2S_2$: C, 56.71; H, 6.19; N, 9.92; S, 15.14. Found: C, 56.91; H, 6.22; N, 9.89; S, 15.10.

Molecular Biology. A cDNA encoding the vanilloid receptor rVR1 was cloned from rat DRG total RNA by reverse-transcription-polymerase chain reaction using primers base on the published nucleotide sequence (Caterina et al., 1997). A 2.7-kilobase cDNA was isolated and the nucleotide sequence was verified to be identical to the published sequence. This cDNA was subcloned into pUG102–3 (BD Biosciences Clontech, Palo Alto, CA).

Stable VR1 Expression Cell Line Preparation and Subculture. The pUHG102–3 rVR1 plasmid was transfected into Chinese hamster ovary (CHO) cells carrying the pTet Off regulatory expression plasmid (BD Biosciences Clontech). In these cells, expression of the pUHG plasmid is repressed in the presence of tetracycline but is induced upon removal of the antibiotic. Stable clones were isolated in

culture medium containing puromycin (10 μg/ml) and maintained in medium supplemented with tetracycline (1 μg/ml) (Szallasi et al., 1999). Cells used for assays were grown in culture medium without antibiotic for 48 h before use. For radioligand binding experiments, cells were seeded in T75 cell culture flasks in media without antibiotics and grown to approximately 90% confluence. The flasks were then washed with PBS and 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 uptake, cells were seeded into 24-well plates and grown to 70 to 90% confluence. For calcium imaging, cells were grown on glass coverslips (25 mm).

DRG Neuron Isolation and Culture. Two- to 3-week-old rats were killed by decapitation under CO_2 anesthesia. The spinal columns were removed aseptically and dorsal root ganglia from all levels were dissected out and collected in DMEM containing 0.5% heat inactivated FBS (Invitrogen, Carlsbad, CA), 1 mM Na-pyruvate, 25 mM HEPES, pH 7.55, and antibiotics. Ganglia were digested with 1 mg/ml collagenase (Sigma-Aldrich, St. Louis, MO) and 0.125 mg/ml trypsin (Sigma-Aldrich) for 30 min at 37°C. The digestion was terminated by addition of 10% FBS. The ganglia were triturated with a flame-polished Pasteur pipette to form a single cell suspension. Cells were then washed three times with DMEM, they were resuspended in the same medium, and the number of viable cells was determined. Cells were plated onto coverslips coated with 10 μg/ml polylysine and were incubated for 24 h in DMEM containing 10% FBS, antibiotics, 200 ng/ml mouse submaxillary gland 2.5 S NGF (Sigma), and 10^{-5} M cytosine arabinoside (Sigma). The medium was then changed to fresh medium lacking the cytosine arabinoside and the cells incubated for a further 2 days before being used for experiments.

Competition Binding Assay. Binding studies with [^{3}H]RTX were carried out as described previously with minor modifications (Szallasi et al., 1992). Binding assay mixtures were set up on ice and contained 40 pM [^{3}H]RTX, various concentrations of competing ligands, 0.25 mg/ml bovine serum albumin (Cohn fraction V), and 5×10^4 to 5×10^5 VR1-transfected cells. The final volume was adjusted to 450 μl with DPBS with Ca^{2+} and Mg^{2+} 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. Membrane-bound RTX was separated from the free by pelleting the membranes in a Beckman 12 (Beckman Coulter, Fullerton, CA) bench-top centrifuge (15 min, maximal velocity), 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 Uptake. CHO/rVR1 cells were incubated for 5 min at 37°C or 44°C with 0.2 μCi of ^{45}Ca 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 uptake, cells were incubated for 5 min at 22°C with 1 μCi ^{45}Ca in the presence of Dulbecco's PBS (DPBS) with Ca^{2+} and Mg^{2+}, supplemented with 0.25 mg/ml bovine serum albumin, adjusted to the indicated pH with 1 M MES (Sigma). After incubation, cells were washed three times with DPBS (with Ca^{2+}, Mg^{2+}), and lysed in 400 μl/well of radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, and 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 acetoxymethyl ester (10 μM)

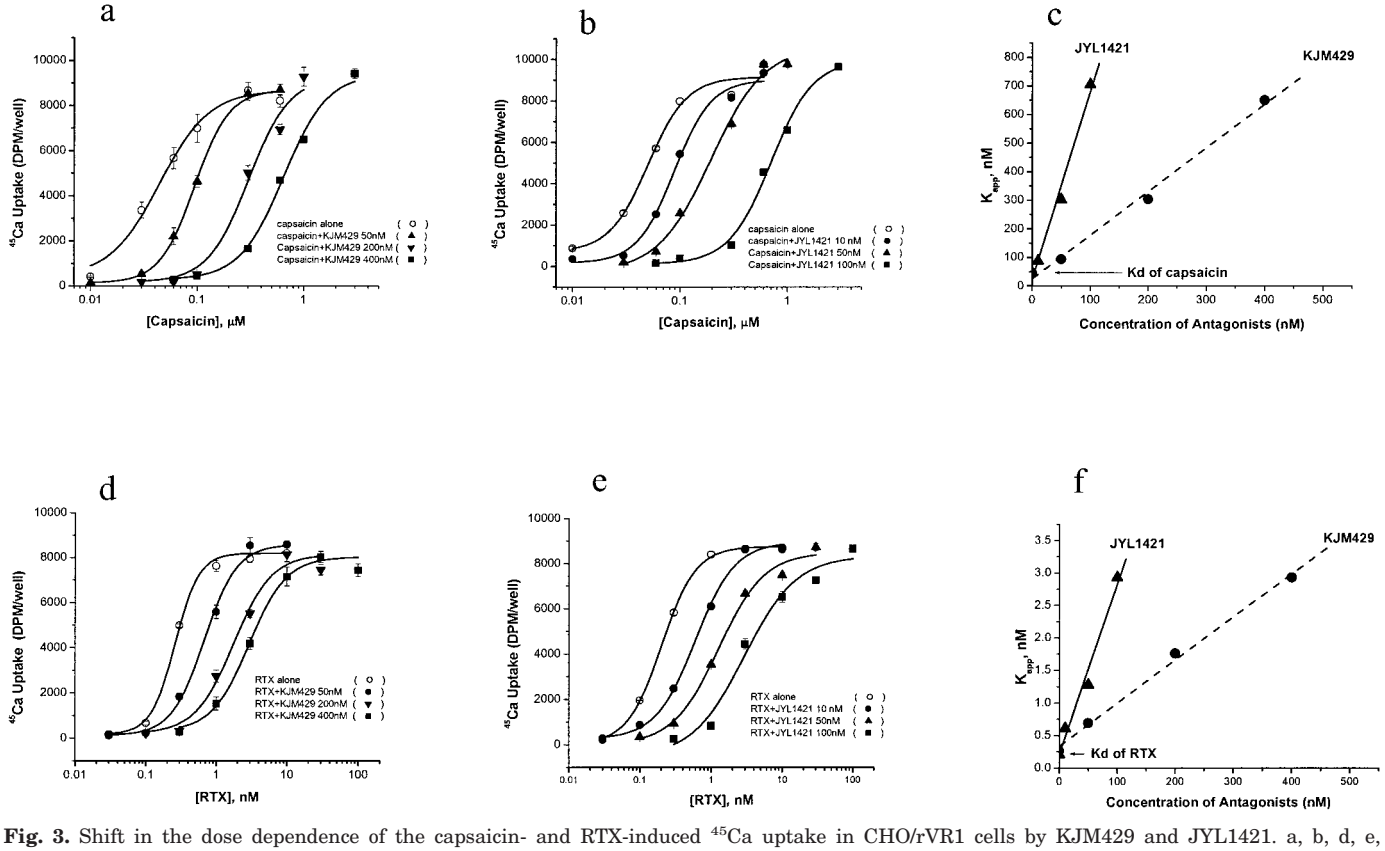


Fig. 3. Shift in the dose dependence of the capsaicin- and RTX-induced ^{45}Ca uptake in CHO/rVR1 cells by KJM429 and JYL1421. a, b, d, e, dose-response curves were determined for agonists alone and in the presence of the indicated doses of antagonists. Points represent mean values of quadruplicate determinations in single, representative experiments; error bars indicate S.E.M. All experiments were repeated at least two additional times with similar results. c, f, plot of K_{app} versus the concentration of KJM429 or JYL1421.

for 10 min at 37°C and an additional 50 min at room temperature (CHO/rVR1 cells) or 30 min at 37°C (DRG neurons), 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 nM and 380 nM were captured using a Cohu 4915 low-light charge-coupled device camera on an InCyt Dual-wavelength Fluorescence Imaging and Photometry System (Intracellular Imaging Inc., OH). After subtraction of background fluorescence, the ratio of fluorescence intensity at the two wavelengths was calculated.

Results

JYL1421 and KJM429 Bind to the Vanilloid Receptor and Antagonize Its Response to Ligands. We have previously demonstrated that ligand binding to rVR1 heterologously expressed in CHO cells or HEK293 cells closely resembles that characterized in dorsal root ganglion neurons (Szallasi et al., 1999). We have therefore routinely used that measure for determination of ligand structure activity relations. KJM429 and JYL1421, a derivative of KJM429 substituted with a fluorine in the *m*-position of the phenyl ring in the A region, inhibited [^{3}H]RTX binding to rVR1 with K_i values of 62.6 ± 10.1 (four experiments) and 53.5 ± 6.5 nM (three experiments), respectively (Fig. 2a). Capsazepine, in contrast, inhibited with a K_i of only 1310 ± 150 nM (three experiments, current studies) (6600 nM; Szallasi et al., 1999) (Fig. 2a). We conclude that KJM429 and JYL1421 are 10 to 25-fold more potent than capsazepine for binding to rVR1.

We evaluated antagonism by the ability of the compounds to inhibit the ^{45}Ca uptake induced by 50 nM capsaicin. KJM429 and JYL1421 inhibited the response of CHO/rVR1 cells with K_i values of 53.9 ± 8.7 nM (four experiments) and 9.2 ± 1.6 nM (three experiments), respectively (Fig. 2b).

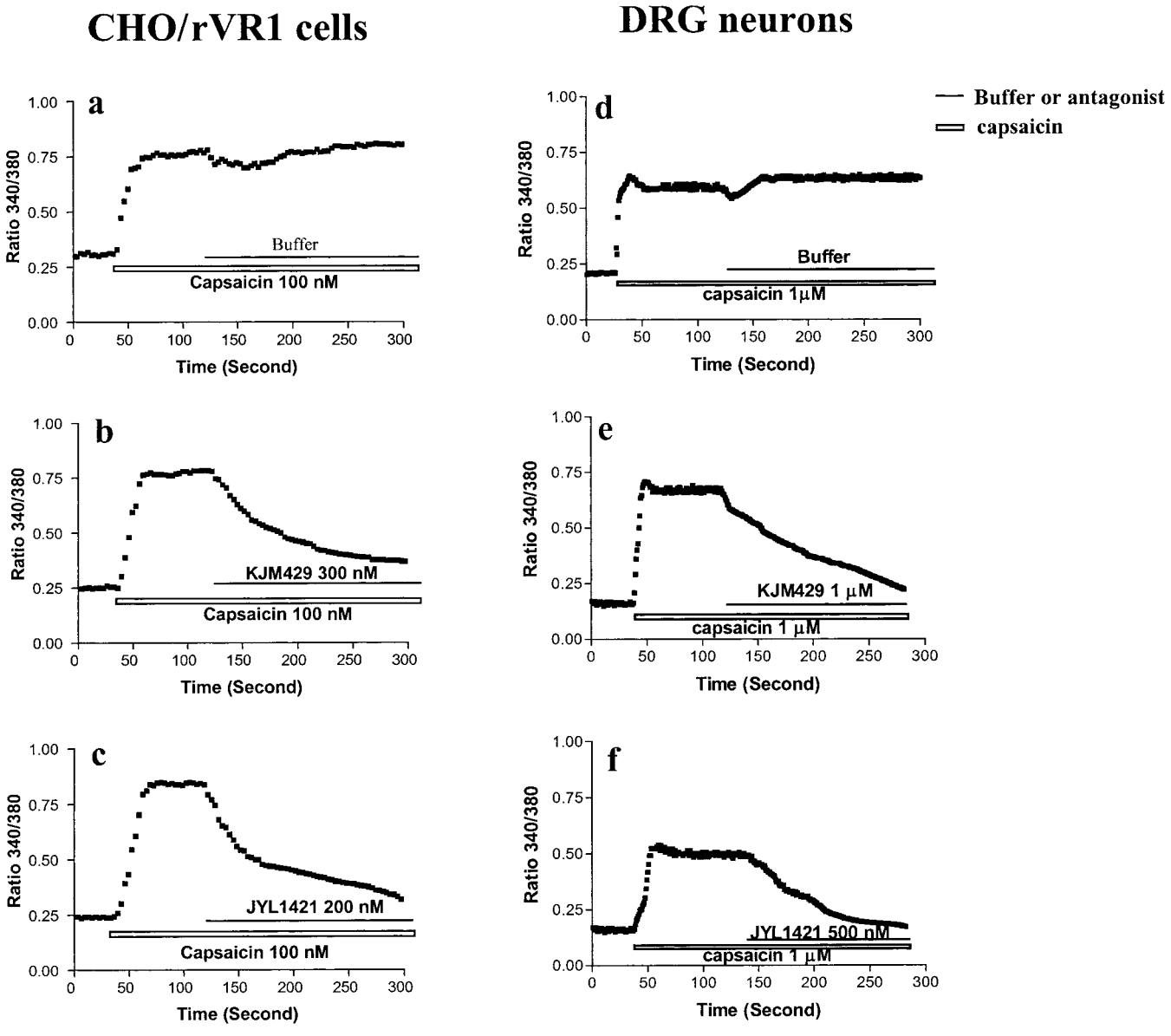


Fig. 4. Antagonism of capsaicin evoked calcium mobilization in CHO/rVR1 cells and cultured rat DRG neurons as determined by calcium imaging. CHO/rVR1 cells or rat DRG neurons were treated with 100 nM capsaicin as indicated for 120 s before application of buffer, KJM429, or JYL1421 (indicated by horizontal line) in the continued presence of capsaicin. 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.

Capsazepine was once again appreciably less potent, with a K_i of 520 ± 12 nM (three experiments) (Fig. 2b). The levels of inhibition under these assay conditions were >98% for all three compounds. We conclude that both KJM429 and JYL1421 function as full antagonists under these conditions. JYL1421 was thus 60-fold more potent than capsazepine; KJM429 was somewhat less so. We further evaluated antagonism by inhibition of the ^{45}Ca uptake induced by 0.26 nM RTX. Results were similar to those with capsaicin, with K_i values of 30.4 ± 5.7 nM (three experiments), 12.4 ± 1.6 nM (three experiments), and 315 ± 24 nM (three experiments), for KJM429, JYL1421, and capsazepine, respectively (Fig. 2c). The antagonism was thus not dependent on our choice of capsaicin as the agonist for induction of ^{45}Ca uptake.

KJM429 and JYL1421 Antagonize ^{45}Ca Uptake Induced by Capsaicin and RTX through a Competitive Mechanism. To test whether KJM429 and JYL1421 were antagonizing vanilloid action through a competitive mechanism, we determined the dose response curves for induction of ^{45}Ca uptake by capsaicin and RTX as a function of the concentration of KJM429 or JYL1421. Cells were incubated with fixed concentrations of antagonist together with varied concentrations of capsaicin for 5 min (Fig. 3, a, b, d, e). For both antagonists, the dose-response curves for induction of ^{45}Ca uptake in CHO/rVR1 cells by both agonists were shifted to the right with no depression of the maximal response. Using the relationship $K_{app} = K_d + (K_d/K_i)$ (I), where K_{app} is the measured ED_{50} in the presence of the antagonist, K_d is the ED_{50} in the absence of antagonist, K_i is the dissociation constant for the antagonist, and I is the concentration of antagonist, we obtained a linear fit between K_{app} and I (Fig. 3, c and f) (Matthews, 1993). We derived K_i values for KJM429 of 31.2 ± 0.8 nM (three experiments) as an antagonist of capsaicin and of 35.2 ± 0.3 nM (three experiments) as an antagonist of RTX. For JYL1421, we obtained K_i values of 8.0 ± 0.3 nM (three experiments) as an antagonist of capsaicin and of 9.6 ± 0.1 nM (three experiments) as an antagonist of RTX. For capsazepine, we obtained K_i values of 430 ± 10 nM (three experiments) as an antagonist against capsaicin and of 460 ± 10 nM (three experiments) as an antagonist against RTX. These values agree well with those obtained from the curves for antagonism at a fixed concentration of agonist as described previously. We conclude that KJM429 and JYL1421 indeed antagonized capsaicin and RTX action through a competitive mechanism.

Antagonism of Capsaicin Action by KJM429 and JYL1421 as Determined by Calcium Imaging Analysis of Both CHO/rVR1 Cells and Cultured Rat DRG Neurons. Using visualization of intracellular calcium with Fura-2 and imaging of individual cells, we determined the ability of KJM429 and JYL1421 to block the calcium uptake induced by capsaicin, both on the cloned rVR1 heterologously expressed in CHO cells (Fig. 4, b and c) as well as of the rVR1 endogenously present in rat DRG neurons (Fig. 4, e and f). Cells were preincubated with capsaicin for 2 min before addition of each antagonist in the continued presence of capsaicin. KJM429 and JYL1421 antagonized the elevation in intracellular calcium in response to capsaicin in both systems. These results confirm our findings with ^{45}Ca uptake. Moreover, they show that the antagonism is not an artifact of the heterologous expression system but is also observed for the rVR1 endogenously present in the rat DRGs. It should be observed, however, that the DRGs were somewhat less sensitive to the antagonists than were the CHO/rVR1 cells. We assume that this reflects the effect of spare receptors, and DRGs have likewise been found to be somewhat less sensitive to other vanilloids for calcium uptake than are heterologous rVR1 overexpressors.

JYL1421 and KJM429 Antagonized Heat-Induced (44°C) Calcium Uptake. Heat represents a second and physiologically relevant class of agonists for activation of VR1. It has been reported that capsazepine was less effective for inhibiting response to heat than response to capsaicin (Nagy and Rang, 1999; Savidge et al., 2001). Using calcium uptake, we examined the ability of KJM429, JYL1421, and capsazepine to block heat-induced (44°C) calcium uptake in CHO/rVR1 cells. Unlike vanilloid stimulated calcium uptake, which was fully antagonized by all three compounds under our assay conditions, heat-induced calcium uptake was differentially antagonized by the three compounds (Fig. 5). JYL1421 was an almost complete antagonist at 300 nM,

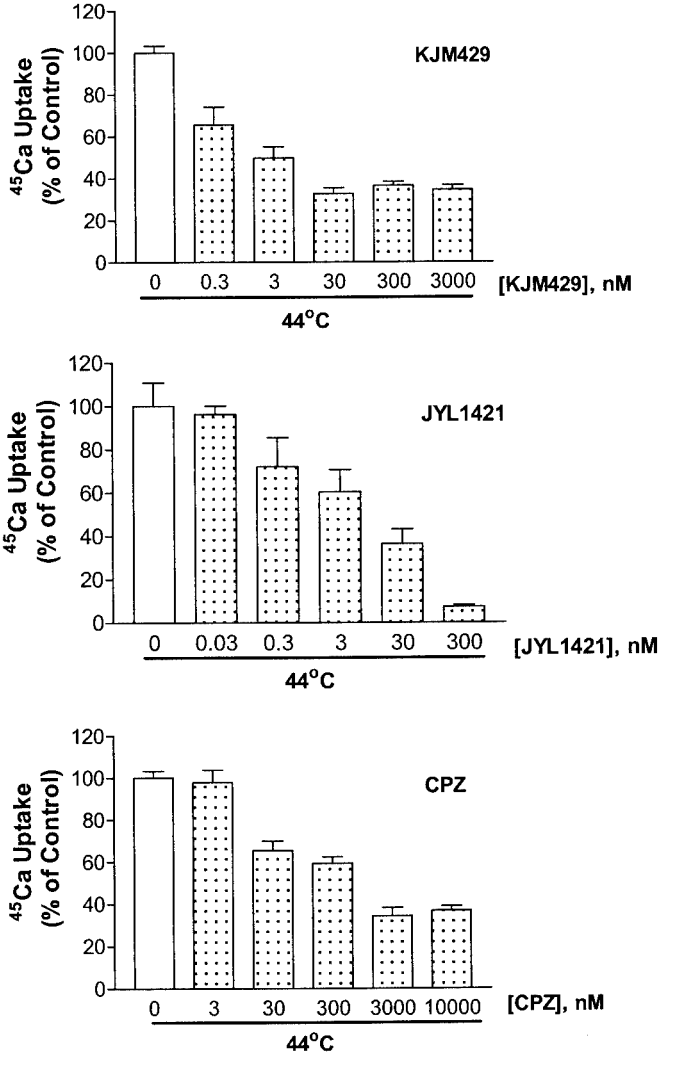


Fig. 5. Inhibition by different antagonists of heat (44°C) induced ^{45}Ca uptake by CHO/rVR1 cells. Points represent mean values of quadruplicate determinations in single, representative experiments; error bars indicate S.E.M. All data were subtracted from baseline (cells incubated at 37°C just with medium) and normalized by the percentage of control (without antagonists group). All experiments were repeated at least two additional times with similar results.

whereas KJM429 inhibited by only 65.4 ± 5.1% (three experiments) and capsazepine inhibited the response by only 64.4 ± 4.5% (three experiments). Secondly, JYL1421 was more potent than KJM429, as previously observed for antagonism of vanilloid-induced calcium uptake, and was markedly more potent than capsazepine.

The Effect of KJM429 and JYL1421 on Proton-Induced Calcium Uptake. Protons represent the third class of well-characterized agonists for VR1 and are of particular interest because of the physiological role that acidosis is believed to play in inflammatory pain. Mutational analysis suggests, moreover, that this response is complex, with multiple residues involved in the proton-coupled activation of the channel. We first compared the ability of KJM429, JYL1421, and capsazepine to antagonize the activation of VR1 by acidification to pH 6.0, as assayed by ^{45}Ca uptake (Fig. 6, a-c). As with activation by heat, JYL1421 functioned as a complete antagonist, whereas KJM429 blocked uptake by 57.8 ± 2.6% (three experiments) and capsazepine reduced uptake by 71.1 ± 5.3% (three experiments) at concentrations of 10 μM. In addition, JYL1421 seemed modestly more potent as an antagonist for pH 6.0-induced activation than it was in the other assays.

Because of the suggested complexity of the pH response, we also compared the ability of KJM429, JYL1421, and capsazepine to antagonize the activation of VR1 by acidification to pH 5.5 (Fig. 6, d-f). At this pH, JYL1421 remained potent but the antagonism became partial, with 63.9 ± 4.6% (three experiments) inhibition of the proton induced response. KJM429, in contrast to JYL1421, showed agonist activity at this pH, and capsazepine remained as a weak, partial antagonist.

These results demonstrate that compounds cannot be regarded simply as antagonists or agonists for rVR1 but rather that their actions can vary depending on the specific stimulus for activation of rVR1. Thus, JYL1421 was a complete antagonist for stimulation of rVR1 by vanilloids, heat, and pH 6.0, whereas KJM421 was only a partial antagonist for the latter two stimuli, although it was a complete antagonist for vanilloids under our assay conditions.

Evaluation of Specificity of Antagonists for rVR1. One of the major deficiencies of capsazepine, in addition to its weak potency, is its inhibitory activity on other channels [e.g., voltage gated calcium channels (Docherty et al., 1997) and the nicotinic cholinergic receptor (Liu and Simon, 1997)]. Using normal CHO cells not transfected with rVR1, we have observed that capsazepine is also able to inhibit ^{45}Ca uptake induced by ATP (Fig. 7). Its potency (K_i of 1090 ± 32 nM;

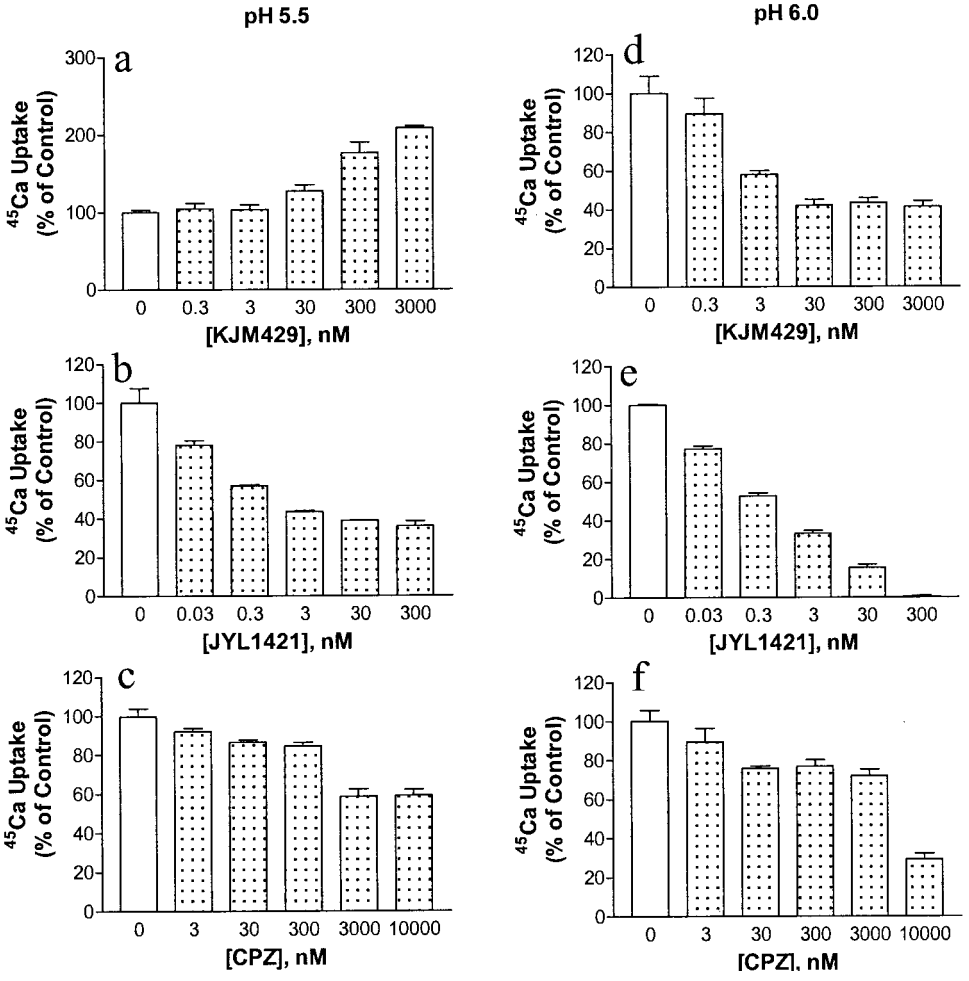


Fig. 6. Effect of different antagonists on proton (pH 6.0 and pH 5.5) induced ^{45}Ca uptake in CHO/rVR1 cells. Points represent mean values of triplicate determinations in single, representative experiments; error bars indicate SEM. All data were relative to baseline (cells incubated at pH 7.0 without antagonist) and normalized by the percentage of control (without antagonists). All experiments were repeated at least two additional times with similar results.

three experiments) was similar to that observed for antagonism of rVR1. In contrast, JYL1421 gave no significant inhibition up to 300 nM ($p > 0.12$, Student's t test) and KJM429 gave only 43.2 ± 6.7% (three experiments) inhibition up to 1 μM, a concentration 20 times its K_i for antagonism of capsaicin. Similar results were obtained by calcium imaging (Fig. 8). Cells were preincubated with each of the antagonists for 2 min before application, in the continued presence of the antagonist, of α,β-methyleneadenosine 5′-diphosphate, a selective agonist of the ATP gated P_2X_3 receptor (Fig. 8). Whereas 10 μM capsazepine partially inhibited the response, no inhibition was observed for 100 nM JYL1421 or KJM429.

Discussion

The pharmacology of VR1 affords great opportunities for exploitation through medicinal chemistry. Recognition has emerged over the past few years that the receptor affords a binding surface that can generate much higher binding affinities than found for capsaicin. As we have described, the natural product RTX, which represents a standard capsaicin analog with the C-region substituted with a tricyclic diterpene, binds to rVR1 with some 4 orders of magnitude higher affinity than does capsaicin (Szallasi et al., 1999). These opportunities had not previously been fully revealed through fairly extensive medicinal chemistry (Walpole et al., 1996a,b,c). Using a strategy seeking to incorporate and constrain some of the functional groups contributed by the diterpene moiety of RTX, we have been able to produce novel vanilloid agonists with several hundred-fold enhancement of binding potencies (Lee et al., 1999, 2001a,b; 2002).

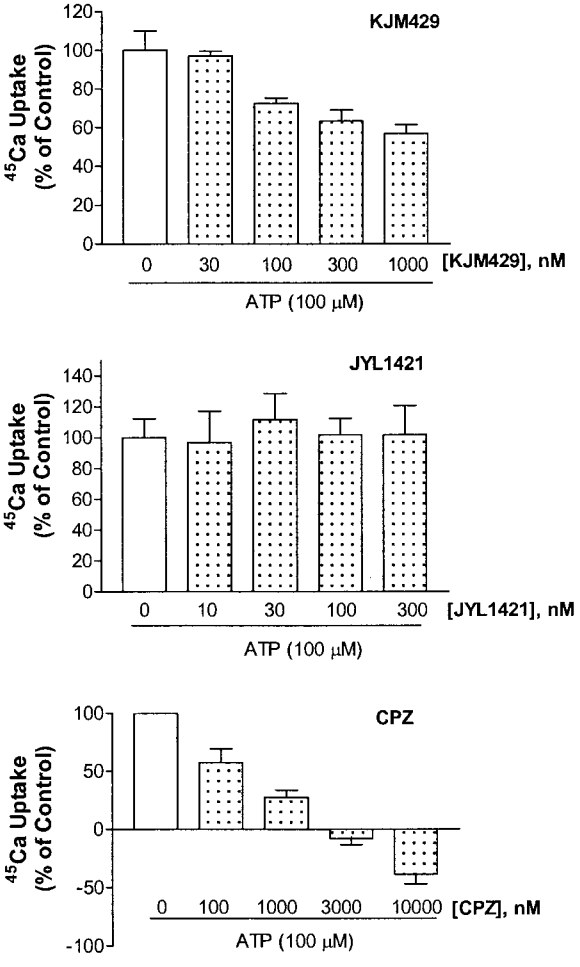


Fig. 7. Effect of different antagonists on ^{45}Ca uptake evoked by treatment with 100 μM ATP of control CHO cells not expressing rVR1. Points represent mean values of quadruplicate determinations in single, representative experiments; error bars indicate S.E.M. All data were relative to baseline (cells incubated without ATP and antagonists) and normalized by the percentage of control (without antagonists group). All experiments were repeated at least two additional times with similar results.

An important finding is that medicinal chemistry can at least in part dissect the different biological endpoints associated with vanilloid action. RTX is much more potent for desensitization than for induction of acute pain in the eye-wiping assay (Szallasi and Blumberg, 1989). Piperine has been reported to be pungent but not to desensitize (Liu and Simon, 1996). Several capsaicin analogs with a phorbol ester as a C region were inactive for modulating thermoregulation, although they were potent for neurogenic inflammation (Szallasi et al., 1989; Appendino et al., 1996). The loss of the range of vanilloid responses in mice null for VR1 argues against the otherwise attractive model that multiple genes for the vanilloid receptor exist (Caterina et al., 2000; Davis et al., 2000). Nevertheless, the vanilloid pharmacology unambiguously demonstrates that there must be mechanisms that can generate from VR1 heterogeneity of response and that can be distinguished by ligand chemistry. Plausible mechanisms include association of VR1 with splice variants (Xue et al., 2001) or other proteins, different subcellular localizations for VR1 (Olah et al., 2001), and modification of VR1 by phosphorylation (Premkumar and Ahern, 2000) or other mechanisms (Kwak et al., 2000). Regardless of mechanism, the whole animal behavior has amply documented the diversity of behavior from a small assortment of compounds. Much opportunity should be afforded by a vigorous medicinal chemical approach.

Building on our previous efforts to design high-affinity vanilloid agonists, we have also been able to develop an extensive series of antagonists for rVR1 (Lee et al., 2001b; 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.-K. Pork, et al., manuscript in preparation). Here, we have characterized in detail two of these antagonists at the cellular level and compared their behavior with that of capsazepine.

At the practical level, a contribution of this study is that we have identified a rVR1 antagonist, JYL1421, which is 25- to 60-fold more potent than capsazepine, the antagonist currently in common use. It thus falls in a similar potency range to 5-iodo-RTX. JYL1421 blocks the activation of rVR1 by both temperature and pH as well as by capsaicin, thus showing a broader range of antagonistic activity than capsazepine. Finally, we might expect that the enhanced potency of JYL1421 would be associated with correspondingly enhanced specificity. We in fact showed that this was true for one non–VR1-mediated response, inhibition of ATP-induced calcium uptake. ATP-induced calcium uptake was blocked by capsazepine at a dose similar to that active on rVR1 but was not blocked by JYL1421 at a dose 30 times that antagonizing rVR1. Naturally, whether JYL1421 has improved specificity relative to either the other secondary targets already characterized for capsazepine or other, as-yet-unidentified targets remains unknown.

At the conceptual level, a finding of our study is that

antagonism for different activators of VR1 is associated with different structure activity requirements. Thus, whereas all three compounds, JYL1421, KJM429, and capsazepine, antagonized capsaicin-induced rVR1 activation, only JYL1421 was a complete antagonist at pH 6.0. Likewise, whereas both JYL1421 and KJM429 antagonized at least partially at pH 6.0, at pH 5.5, KJM429 acted as a partial agonist rather than as an antagonist. This issue is important because it is not yet clear which class(es) of agonist is most relevant from the perspective of therapeutic development. Endogenous ligands for VR1 include anandamide or lipoxygenase products (Hwang et al., 2000; Smart and Jerman, 2000). Likewise, tissue acidification associated with inflammation could represent a relevant activation mechanism (Olah et al., 2001; Voilley et al., 2001). Although antagonism of VR1 activation by capsaicin may be a convenient initial screening approach, its evaluation against the range of activators for VR1 is needed for appropriate assessment of its utility.

In principle, the divergence in structure activity for different classes of activators is not unexpected. Antagonism of capsaicin action will result from binding to the capsaicin-binding site as long as the compound does not induce the conformational change associated with channel opening. Antagonism against temperature or pH, in contrast, will depend on the ability of the bound compound to suppress the open channel conformation. It is, moreover, not surprising that different pH values may behave differently in response to antagonists. Careful molecular analysis of residues involved in proton regulation of VR1 suggests several residues with different effects: an extracellular Glu residue (E600) serves as an important regulatory site for proton potentiation of vanilloid receptor activity over a physiologically relevant range (pH 6–8), whereas mutations at a second extracellular site (E648) significantly reduce proton activated currents without altering heat- or capsaicin-evoked responses or without eliminating the ability of protons to potentiate responses to these stimuli (Jordt et al., 2000).

Consistent with the finding that VR1 channel opening in response to different agonists, namely capsaicin, heat, and pH, can be associated with different dependence on antagonist structure is our previous observation that minor changes in structure can shift the activity of a ligand from agonist to antagonist (Lee et al., 2001b). For example, the replacement of benzoate with 3,4-dimethylbenzoate in the antagonist 2-benzyl-3-{[(6,7-dihydroxy-3,4-dihydro-2(1H)-isoquinolinyl) carbothioyl]amino}propyl benzoate converts the compound into an agonist (Lee et al., 2001b). Similarly, 2-iodo-RTX was described as an agonist for human VR1 with 75% of the

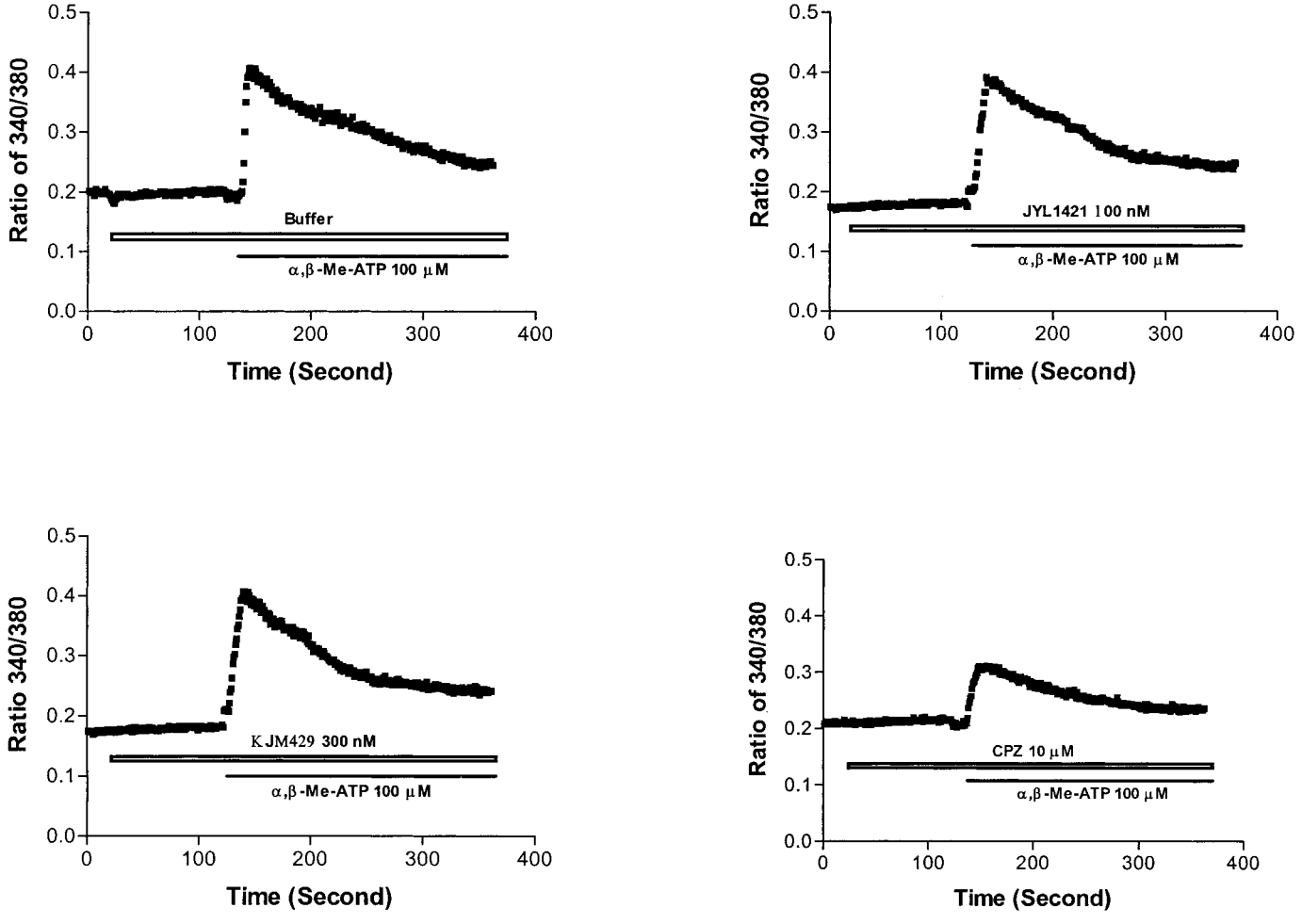


Fig. 8. Effect of different antagonists on calcium mobilization evoked by 100 μM α, β-Me-ATP in control CHO cells, not expressing rVR1, and detected using calcium imaging. Cells were pretreated with buffer or antagonist for 120 s before application of α,β-methyleneadenosine 5′-diphosphate (α,β-Me-ATP) in the continued presence of the buffer or antagonist. 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.

efficacy of capsaicin, whereas 5-iodo-RTX was an antagonist (McDonnell et al., 2002). In the present study, the difference in behavior of JYL1421 compared with KJM429 reflects merely the m-fluoro substitution on the phenyl ring of the A-region.

RTX has provided an attractive tool for the assessment of the therapeutic utility of C-fiber desensitization. Together with 5-iodo-RTX, JYL1421 represents a step in the development of potent, specific VR1 antagonists. Capsazepine has been reported to inhibit induction of fos in the spinal cord in response to peripheral inflammation (Kwak et al., 1998), consistent with a role for endogenous activators of VR1 in response to inflammation. The *N*-alkylglycyl trimers active as VR1 channel blockers attenuated response of mice to noxious heat, thermal hyperalgesia in response to mustard oil, and neurogenic inflammation in response to capsaicin (Garcia-Martinez et al., 2002). Compounds such as JYL1421 or 5-iodo-RTX may facilitate detailed assessment of the therapeutic utility of antagonists.

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Correction to "High affinity antagonists of the vanilloid receptor"

In the above article [Wang Y, Szabo T, Welter JD, Toth A, Tran R, Lee J, Kang SU, Suh YG, Blumberg PM, and Lee J (2002) *Mol Pharmacol* **62:**947–956], please note the following corrections.

Some authors were inadvertently omitted from the article. The complete and correct list of authors and affiliations is as follows:

Yun Wang, Tama Szabo, Jacqueline D. Welter, Attila Toth, Richard Tran, Jiyoun Lee, Sang Uk Kang, Yong-sil Lee, Kyung Hoon Min, Young-Ger Suh, Mi-kyung Park, Hyeung-geun park, Young-Ho Park, Hee-Doo Kim, Uhtaek Oh, Peter M. Blumberg, and Jeewoo Lee

National Cancer Institute, Bethesda, Maryland (Y.W., T.S., J.D.W., A.T., R.T., P.M.B.); College of Pharmacy, Seoul National University, Seoul, Korea (Ji.L., S.U.K., Y.L., K.H.M., Y.-G.S., M.P., H.P., U.O., Je.L.), Sookmyung Women's University, Seoul, Korea (H.-D.K.); Amore Pacific Corp., R&D Center, Yongin, Korea (Y.-H.P.)

On pages 948 (left column, last line) and 954 (right column, line 38), patents WO 02/16317, WO 02/16318, and WO 02/16319 should be cited as references for the series of derivatives that function as rVR1 antagonists. Full information for these patents is provided below:

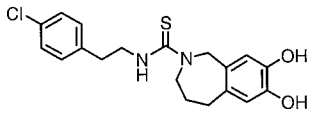
Suh YG, Oh UT, Kim HD, Lee, JW, Park HG, Park YH, and Yi JB (2002) inventors; Pacific Corporation, assignee. Novel thiocarbamic acid derivatives and the pharmaceutical compositions containing the same. International patent WO 02/16317 A1. 2002 Feb 28.

Suh YG, Oh UT, Kim HD, Lee JW, Park HG, Park OH, Lee YS, Park YH, Joo YH, Choi JK, Lim KM, Kim SY, Kim JK, Koh HJ, Moh JH, Jeong YS, Yi JB, and Oh YI (2002) inventors; Pacific Corporation, assignee. Novel thiourea derivatives and the pharmaceutical compositions containing the same. International patent WO 02/16318 A1. 2002 Feb 28.

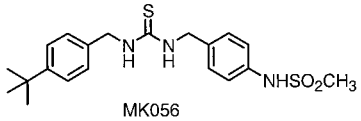
Suh YG,Oh UT, Kim HD, Lee JW, Park HG, Park YH, Yi JB (2002) inventors; Pacific Corporation, assignee. Novel thiourea compounds and the pharmaceutical compositions containing the same. International patent WO 02/16319 A1. 2002 Feb 28.

Finally, KJM429 and JYL1421 correspond to the compound designations MK056 and SC0030 (Fig. 1) in the above patents.

The authors regret these errors and apologize for any confusion or inconvenience they may have caused.



Capsazepine



MK056

SC0030

Fig. 1.