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Binding of [^{3}H]A-778317 to native transient receptor potential vanilloid-1 (TRPV1) channels in rat dorsal root ganglia and spinal cord

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ABSTRACT

A-778317 (1-((R)-5-*tert*-butyl-indan-1-yl)-3-isoquinolin-5-yl-urea) is a potent antagonist of human and rat transient receptor potential vanilloid-1 (TRPV1) receptors. We have previously reported that [^{3}H]A-778317 is an excellent radioligand to study the recombinant human TRPV1 receptor in a heterologous expression system. These studies were extended to determine the feasibility of using [^{3}H]A-778317 to label native TRPV1 channels in rat tissues. Saturable high-affinity binding of [^{3}H]A-778317 was detected in membrane preparations of rat dorsal root ganglia (DRG) and spinal cord that was inhibited by TRPV1 receptor agonists and antagonists. [^{3}H]A-778317 labeled a single class of high-affinity binding sites in both rat DRG and spinal cord membranes ($K_D = 10$ and 8.4 nM, respectively). The number of binding sites was 10-fold higher in rat DRG membranes than spinal cord membranes ($B_{max} = 3.3$ and 0.35 pmol/mg protein, respectively). The pharmacology of the high-affinity binding sites was similar in rat DRG and spinal cord, but differed from the recombinant rat TRPV1 (rTRPV1) receptor expressed in transiently transfected HEK293-F cells. In particular, a large disparity in potency (>300-fold) was observed for the TRPV1 receptor agonist resiniferatoxin between native and recombinant rTRPV1 receptors. Our data indicate that the binding of [^{3}H]A-778317 to native rTRPV1 channels is pharmacologically distinct, and perhaps more complex, than its binding to the recombinant rTRPV1 receptor.

1. Introduction

The involvement of the transient receptor potential vanilloid-1 (TRPV1) receptor in the transmission and modulation of pain signals in the periphery is well-documented (Szallasi and Blumberg, 1999; Caterina and Julius, 2001). The TRPV1 receptor is a nonselective cation channel that can be activated by diverse painful stimuli, such as heat (>43 °C), protons (<pH 6) and capsaicin, and also by endogenous ligands such as N-arachidonoyl-dopamine (NADA) (Huang et al., 2002), arachidonylethanolamide (anandamide) (Smart et al., 2000) and certain lipoxygenase products (12- and 15-(S)-hydroperoxyeicosatetraenoic acids) (Hwang et al., 2000). The TRPV1 channel is highly expressed in nociceptive neurons of the dorsal root and trigeminal ganglia (Mezey et al., 2000; Sanchez et al., 2001), which transmit pain signals from the periphery to the spinal cord. Expression of the TRPV1 channel in the spinal cord has been detected in sensory nerve terminals located in lamina I (presynaptic), and also postsynaptically in lamina II, but at significantly lower levels than sensory ganglia (Valtschanoff et al., 2001).

1-((R)-*tert*-butyl-indan-1-yl)-3-isoquinolin-5-yl-urea (A-778317) is a small molecule TRPV1 receptor antagonist that is similar in structure to 1-isoquinolin-5-yl-3-(4-trifluoromethyl-benzyl)-urea (A-425619)(El Kouhen et al., 2005; Bianchi et al., 2007a). A-778317 has the same effect on TRPV1 function as A-425619, and is able to fully block the activation of the TRPV1 receptor by capsaicin, NADA, anandamide, protons and heat. It also shows stereoselectivity for the TRPV1 receptor over its weaker enantiomer 1-((S)-*tert*-butyl-indan-1-yl)-3-isoquinolin-5-yl-urea (A-778316). We previously reported that a tritiated form of A-778317 with high specific activity ([^{3}H]A-778317; 29.3 Ci/mmol) is an excellent radioligand to study the recombinant human TRPV1 (hTRPV1) receptor in a heterologous expression system (Bianchi et al., 2007a). A binding assay for the hTRPV1 receptor was developed, using [^{3}H]A-778317 as the radioligand, that was less time-consuming, less labor-intensive, and accompanied by lower nonspecific binding, than earlier binding assays using [^{3}H]resiniferatoxin (Szallasi et al., 1999) or [^{125}I]iodoresiniferatoxin (Wahl et al., 2001).

In the present paper we extended our studies to determine whether [^{3}H]A-778317 can selectively label native TRPV1 channels in rat tissues. Electrophysiological studies have previously revealed that A-778317 is a potent antagonist of the native TRPV1 receptor expressed in rat dorsal root ganglia (DRG), and effectively reduces both capsaicin (1 μM) and acid (pH 5.5)-evoked inward currents in cultured rat DRG neurons (Bianchi et al., 2007a). Hence, we

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investigated the binding of [^{3}H]A-778317 to the native TRPV1 channels in rat DRG and spinal cord. Saturable and reversible high-affinity binding of [^{3}H]A-778317 was detected in both rat DRG and spinal cord membranes. To determine whether the high-affinity binding was occurring to native TRPV1 channels, the pharmacology of the high-affinity binding was characterized by measuring the ability of TRPV1 agonists and antagonists to inhibit the binding of the radioligand, and then compared to the pharmacology of the recombinant rat TRPV1 (rTRPV1) receptor.

2. Materials and methods

2.1. Materials

Capsaicin and capsazepine were purchased from Sigma-Aldrich (St. Louis, MO), while resiniferatoxin and tinyatoxin were purchased from LKT Laboratories (St. Paul, MN). N-vanillyloleolyamide (olvanil) was purchased from Tocris Bioscience (Ellisville, MO). A-778317, its enantiomer A-778316, A-425619 and 3′-trifluoromethyl-3,6-dihydro-2*H*-[1,2′]bipyridinyl-4-carboxylic acid (4-trifluoromethanesulfonyl-phenyl)-amide (A-784168) (Cui et al., 2006) were synthesized at Abbott Laboratories (Abbott Park, IL). A high specific activity form of [^{3}H]A-778317 (29.3 Ci/mmol; 0.4 Ci/ml) was synthesized as described previously (Bianchi et al., 2007a). Fetal bovine serum, dimethylsulfoxide (DMSO), N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES), Trizma pre-set crystals, pH 7.4, and bovine serum albumin (minimum 96%)(fraction V) were purchased from Sigma-Aldrich (St. Louis, MO). Fluo-4 AM was purchased from Invitrogen Molecular Probes (Eugene, OR). Six to eight day-old Sprague–Dawley rats were obtained from Charles River Laboratories International (Wilmington, MA). Rat spinal cords were obtained from Pel-Freez Biologicals (Rogers, AK).

2.2. Large-scale transient transfection

The rTRPV1 receptor was expressed in FreeStyle HEK293-F cells using the FreeStyle 293 expression system (Invitrogen, Grand Island, NY). The procedure for large-scale transient transfection of the FreeStyle HEK293-F cells was described previously (Bianchi et al., 2007b). 1 mg plasmid DNA was diluted in 35 ml Opti-MEM I (reduced serum medium), and then added to 35 ml Opti-MEM I containing 1.3 ml 239 fectim transfection reagent. The mixture was incubated for 30 min at room temperature to make the DNA-293fectin complex. The DNA-293fectin complex was added to 1 L cell suspension (10^9 cells) in FreeStyle 293 expression medium, and then the cells were incubated for 48 h at 37 °C under a humidified 8% CO_2 atmosphere. Afterward, the cells were harvested by low-speed centrifugation (1000×*g* for 5 min) and resuspended in freezing medium (Freestyle 293 expression medium containing 10% (v/v) fetal bovine serum and 10% (v/v) DMSO) at a cell density of 1.5×10^7 cells/ml. Aliquots of the cells (2 ml in cryovials) were slowly frozen to −80 °C in a Nalgene Mr. Frosty slow-freeze device (Sigma-Aldrich, St. Louis, MO) and stored at −80 °C.

2.3. Preparation of membranes

P2 membrane fractions were prepared as described previously (Bianchi et al., 2007a), with minor modifications. DRG were dissected from six to eight day-old Sprague–Dawley rats in accordance with the guidelines and approval of the Institutional Animal Care and Use Committee (IACUC), and stored at −80 °C. Rat spinal cords were shipped frozen by the vendor (Pel-Freez Biologicals, Rogers, AK), and also stored at −80 °C upon arrival. Frozen tissue was thawed on ice and homogenized in ice-cold 10 mM HEPES buffer, pH 7.8, containing 0.32 M sucrose (20 ml per g wet weight tissue) using a motor-driven glass-teflon homogenizing vessel (10 up-and-down strokes). The homogenate was then centrifuged at low speed (1000×*g* for 10 min) at 4 °C to separate membranes from high molecular weight debris. The resulting pellet (P1 fraction) was rehomogenized (in the same volume) and centrifuged again at low speed. The supernatants from the two low-speed spins were combined and centrifuged at high-speed (20,000×*g* for 60 min) to pellet the membranes (P2). The P2 membrane fraction was resuspended in ice-cold 50 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) buffer, pH 7.4. The protein concentration was determined using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Volume was adjusted with ice-cold 50 mM Tris–HCl buffer, pH 7.4, to obtain 50 μg protein per 90 μl (rat DRG membranes) or 100 μg protein per 90 μl (rat spinal cord membranes). Membranes were stored at −80 °C until ready to use.

A similar procedure was used to prepare a P2 membrane fraction of the rTRPV1-expressing HEK293-F cells, with one additional step before homogenization. The cells were rapidly thawed in a 37 °C water bath, resuspended in FreeStyle 293 expression medium (10 ml per aliquot) and transferred to a 50 ml polypropylene centrifuge tube. A low-speed centrifugation step was then performed (1000×*g* for 5 min) to pellet the cells and remove the freezing medium.

2.4. Radioligand binding

The binding of [^{3}H]A-778317 to native rTRPV1 channels was studied in rat DRG and spinal cord membranes. The binding properties of [^{3}H]A-778317 at the recombinant rTRPV1 receptor were determined in membranes prepared from rTRPV1-expressing HEK293-F cells. Frozen membranes were thawed and rehomogenized using a Polytron tissue homogenizer (setting 6 for 5 s) (Brinkmann Instruments, Westbury, NY). 10 mM stock solutions of the test compounds were prepared in DMSO, and then diluted in distilled water to (10×) final concentrations. 90 μl membrane suspension was added to 12×75 mm polypropylene round-base tubes (Sarstedt, Newton, NC) containing 90 μl radioligand solution (2.22×) (in 50 mM Tris–HCl buffer, pH 7.4, containing 2.22 mg/ml bovine serum albumin) and 20 μl test compound solution (10×). The amount of membrane protein added per assay tube was 50 μg for rat DRG and rTRPV1 (transient transfection) membranes, and 100 μg for rat spinal cord membranes. Final assay volume was 200 μl. Ligand-saturation experiments were carried out using 8 to 11 different concentrations of [^{3}H]A-778317 (ranging from 0.4 to 40 nM). A single radioligand concentration of 5 nM was used in ligand-competition binding experiments. Nonspecific binding was defined in the presence of 10 μM A-425619, a potent TRPV1 receptor antagonist structurally related to A-778317 (El Kouhen et al., 2005). All assay tubes were incubated at 25 °C for a minimum of 1 h, and then reactions were terminated by rapid filtration using a 48-well Brandel cell harvester (Model M-48) (Brandel, Gaithersburg, MD). Membranes were trapped on Whatman glass fiber filter paper (GF/B, fired), and washed three times with 5 ml ice-cold 50 mM Tris–HCl buffer, pH 7.4, to remove unbound radioactivity. Filter circles were punched out into mini Poly-Q scintillation vials (Beckman Coulter, Fullerton, CA). 5 ml EcoLume LSC (MP Biomedicals, Inc., Aurora, OH) was added to the vials, and then the vials were capped and incubated overnight at room temperature. The bound tritium radioactivity (dpm) was counted in a Beckman LS6500 scintillation counter. The concentration–effect data was analyzed using GraphPad Prism (GraphPad Software, San Diego, CA). For ligand-saturation experiments, the specific bound counts (dpm) were calculated by subtracting nonspecific from total bound counts, and then the specific bound counts were converted to pmol/mg protein. The bound pmol/mg protein was plotted against the free radioligand concentration (nM), and curve-fit to a one-site binding (hyperbola) equation to derive the dissociation constant of the radioligand (K_D) and the number of binding sites (B_{max}). For ligand-competition binding experiments, the specific bound counts (dpm) were expressed as a percentage of the counts observed in absence of

test compound. The % bound was plotted against the log of the test compound concentration (M), and curve-fit to a four-parameter logistic Hill equation to derive the potency (IC_{50}) of the test compound. The dissociation constant (K_i) of the test compound was then calculated from the Cheng–Prusoff equation: $K_i = IC_{50}/(1+([L]/K_D))$ where [L] is the concentration of the radioligand (Cheng and Prusoff, 1973).

2.5. Ca^{2+} influx assay

The functional potencies of TRPV1 receptor agonists and antagonists were determined in a Ca^{2+} influx assay for the rTRPV1 receptor. Frozen aliquots of rTRPV1-expressing HEK293-F cells were quickly thawed in a 37 °C water bath. The cells were resuspended in FreeStyle 293 expression medium (10 ml per aliquot), transferred to a 50 ml polypropylene centrifuge tube, and pelleted by low-speed centrifugation (1000×g for 5 min). The cells were resuspended in FreeStyle 293 expression medium at a cell density of 5×10^5 cells/ml, and plated out in black 96-well tissue culture plates (with clear bottoms) (Costar, Corning, NY) on the day before the assay (5×10^4 cells per well). The plates were kept in a 37 °C, 5% CO_2/95% air, humidified cell culture incubator until use. On the day of the assay, the medium was removed, and the cells were preloaded with the fluorescent Ca^{2+} chelating dye fluo-4 AM (2 μM in assay buffer) at 25 °C for 2 h. Assay buffer was Dulbecco's phosphate-buffered saline (D-PBS), pH 7.4, containing Ca^{2+}, Mg^{2+}, and 1 mg/ml D-glucose (Ref. 14287; Invitrogen, Grand Island, NY). After loading of the dye was completed, the cells were washed using a MultiWash Advantage multiplate washer (Model 8070-16) (Tricontinent, Suffolk, UK). 10 mM stock solutions of the test compounds were prepared in DMSO, and then diluted in assay buffer to (4×) final concentrations. Test compound solutions were added to the cells at a delivery rate of 50 μl/s. For agonist experiments, a single addition of the agonists (50 μl) was made at the 10 s time point of the experimental run. For antagonist experiments, antagonists were added at the 10 s time point of the experimental run, and then after a 3 min wait, a second addition was made with the TRPV1 receptor agonist capsaicin (10 nM final concentration) to activate the rTRPV1 receptor. Final assay volume for both the agonist and antagonist experiments was 200 μl. Changes in fluorescence were recorded using a fluorometric imaging plate reader (FLIPR) (Molecular Devices, Sunnyvale, CA) ($\lambda_{EX}=488$ nm, $\lambda_{EM}=540$ nm). The concentration–effect data was analyzed using GraphPad Prism (GraphPad Software, San Diego, CA). For agonists, the peak increase in fluorescence over baseline was calculated, and expressed as a percentage of the maximal response to capsaicin. For antagonists, the peak increase in fluorescence over baseline was expressed as a percentage of the control response to capsaicin (in absence of antagonist). The % values were plotted against the log of the test compound concentration (M), and curve-fit to a four-parameter logistic Hill equation to derive EC_{50} and IC_{50} values.

3. Results

3.1. A-778317 is a potent antagonist of the recombinant rTRPV1 receptor

The prototypical TRPV1 receptor agonist capsaicin was found to robustly increase the intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) of rTRPV1-expressing HEK293-F cells in a dose-dependent manner ($EC_{50}=8.4$ nM) (Fig. 1A, Table 1). Three other classic vanilloid agonists (resiniferatoxin, tinyatoxin and olvanil) were also tested, and all were found to increase $[Ca^{2+}]_i$ with efficacy equal to capsaicin. The potencies of resiniferatoxin and tinyatoxin in the Ca^{2+} influx assay were slightly higher than capsaicin ($EC_{50}=4.1$ nM and 4.2 nM, respectively), whereas olvanil was less potent than capsaicin ($EC_{50}=16.0$ nM). No response to capsaicin was observed in mock-transfected cells (data not shown). A-778317 was a potent antagonist

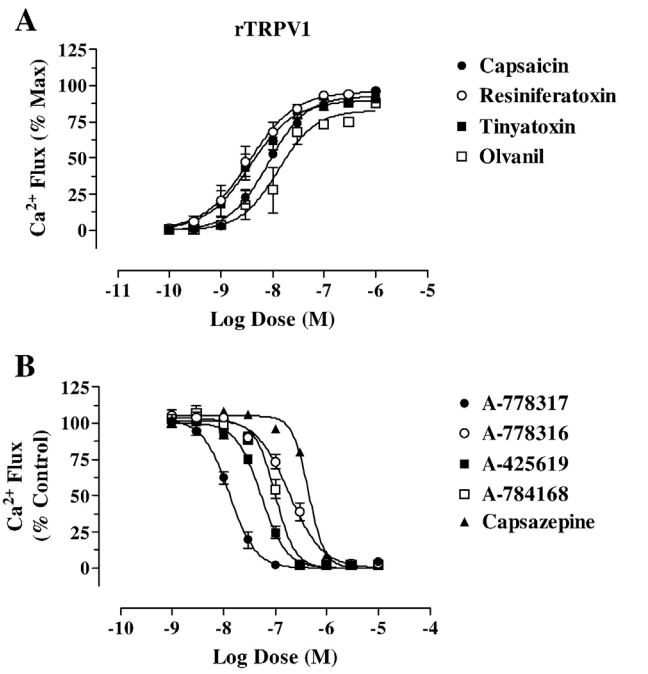

Fig. 1. Concentration–effect curves of TRPV1 receptor agonists and antagonists in a Ca^{2+} influx assay for the recombinant rTRPV1 receptor. A, Dose-dependent stimulation of Ca^{2+} flux by TRPV1 receptor agonists in rTRPV1-expressing HEK293-F cells. Data are expressed as a percentage of the maximal response to capsaicin, and represent the means ± S.E.M. for three or more determinations. B, Concentration–inhibition curves of TRPV1 receptor antagonists. Capsaicin (10 nM)-evoked Ca^{2+} flux was inhibited by A-778317, its weaker enantiomer A-778316, and other TRPV1 receptor antagonists in a dose-dependent manner. Data are expressed as a percentage of the control response to capsaicin, and represent the means ± S.E.M. for three or more determinations.

of the recombinant rTRPV1 receptor, and completely inhibited the response to 10 nM capsaicin in the Ca^{2+} influx assay ($IC_{50}=14.3$ nM) (Fig. 1B, Table 1). A-778317 was approximately 14-fold more potent than its weaker enantiomer A-778316 ($IC_{50}=196$ nM). Three other TRPV1 receptor antagonists were also tested (A-425619, A-784168 and capsazepine), and all completely blocked the response to 10 nM capsaicin. Rank order potency of the TRPV1 antagonists was A-778317>A-425619>A-784168>A-778316>capsazepine.

The concentration–effect curves of capsaicin and resiniferatoxin were shifted to the right in the presence of A-778317 without loss of efficacy (Fig. 2A and B). A Schild plot of the shift in potency of capsaicin (log (dose ratio − 1)) as a function of the A-778317 concentration (log [A-778317] (M)) fits a straight line with a slope of 0.70 ± 0.05

Table 1
Functional potencies of TRPV1 agonists and antagonists in a Ca^{2+} influx assay for the recombinant rTRPV1 receptor.

Compound	Ca^{2+} flux
Agonists	EC_{50} (nM)
Resiniferatoxin	4.1 ± 2.1
Tinyatoxin	4.2 ± 1.9
Capsaicin	8.4 ± 1.2
Olvanil	16.0 ± 7.2
Antagonists	IC_{50} (nM) (against 10 nM capsaicin)
A-778317	14.3 ± 2.4
A-425619	57.6 ± 6.8
A-784168	90.1 ± 15.3
A-778316	196 ± 30
Capsazepine	451 ± 18

Data are shown as means ± S.E.M. for three or more determinations.

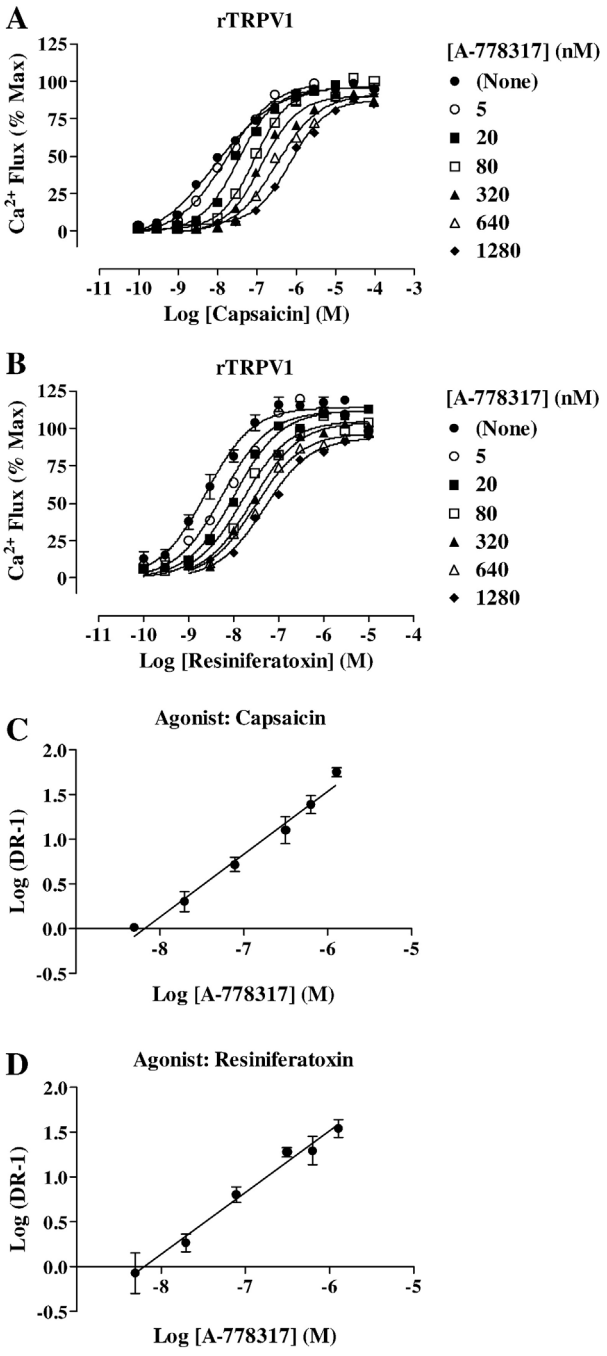

Fig. 2. Schild analyses of A-778317. The concentration–effect curves of capsaicin and resiniferatoxin in a Ca^{2+} influx assay for the recombinant rTRPV1 receptor were shifted to the right in the presence of A-778317 without loss of efficacy. A and B, Concentration–effect curves of capsaicin and resiniferatoxin in the absence and presence of increasing concentrations of A-778317. Data are expressed as a percentage of the maximal response to capsaicin, and represent the means ± S.E.M for three determinations. C and D, Schild plots of the data depicted in panels A and B, respectively. The pA_2 value for A-778317 did not differ between the two Schild plots; $pA_2 = 6.5$ nM when the agonist was capsaicin and 6.3 nM when the agonist was resiniferatoxin.

and goodness of fit $r^2 = 0.91$ (Fig. 2C), indicating that A-778317 is a competitive antagonist of capsaicin at the recombinant rTRPV1 receptor. Schild analysis also indicated that A-778317 is a competitive antagonist of resiniferatoxin at the recombinant rTRPV1 receptor (slope = 0.68 ± 0.06; goodness of fit $r^2 = 0.87$) (Fig. 2D). The pA_2 value for A-778317 (*X*-intercept) was the same whether capsaicin or resiniferatoxin was used as the agonist (6.5 and 6.3 nM, respectively).

3.2. Binding of [^{3}H]A-778317 to the recombinant rTRPV1 receptor

[^{3}H]A-778317 was found to bind to rTRPV1 (transient transfection) membranes with high-affinity. The specific binding of [^{3}H]A-778317 was saturable and achieved a plateau with increasing concentrations of the radioligand (Fig. 3A). The apparent K_D of the radioligand, derived from the saturation binding data, was 8.17 ± 0.37 nM ($n = 3$) (Table 2). The B_{max} was 21.8 ± 0.5 pmol/mg protein ($n = 3$). A Scatchard plot of the saturation binding data was monophasic, a result consistent with binding of [^{3}H]A-778317 to a single class of high-affinity binding sites (Fig. 3B). A small amount of high-affinity binding was also detected in mock-transfected membranes (apparent $K_D = 5.93$ nM and $B_{max} = 2.43$ pmol/mg protein) (Fig. 3B). Specific binding of 5 nM [^{3}H]A-778317 to the mock-transfected membranes comprised about 10% of the specific bound counts in rTRPV1 (transient transfection) membranes. The nature of this binding is not known, but likely involves a protein natively expressed by the host HEK293-F cells. Preheating the mock-transfected membranes briefly in a boiling water bath abolished the binding (data not shown). Nonspecific binding of 5 nM [^{3}H]A-778317 comprised about 10% of the total bound counts.

Both TRPV1 receptor agonists and antagonists inhibited the specific binding of 5 nM [^{3}H]A-778317 to the cell membranes in a dose-dependent manner. Of the TRPV1 receptor agonists tested, resiniferatoxin was the most potent ($K_i = 17.5$ nM) (Fig. 4A, Table 3). However, resiniferatoxin only partially inhibited the specific binding of the radioligand (80%). Tinyatoxin was slightly less potent than

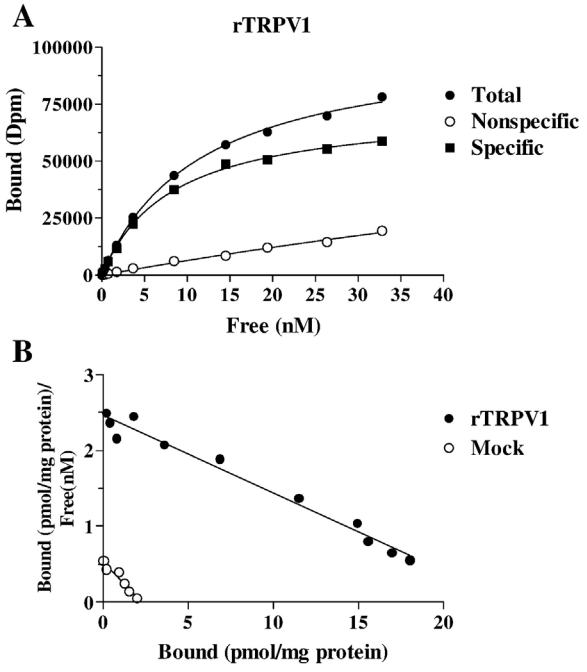

Fig. 3. Saturation binding of [^{3}H]A-778317 to rTRPV1 (transient transfection) membranes. The amount of membrane protein in the binding assay was 50 μg. A, Representative saturation binding curves. Total bound counts (dpm) (filled circles) and nonspecific bound counts (dpm) (open circles) are shown for 11 different radioligand concentrations. Specific bound counts (dpm) (filled squares) were calculated by subtracting nonspecific from total bound counts. Specific binding of [^{3}H]A-778317 was saturable. Mean B_{max} of the specific binding was 21.8 ± 0.5 pmol/mg protein ($n = 3$). B, Scatchard plots of the specific binding of [^{3}H]A-778317 to rTRPV1 (transient transfection) membranes (filled circles; corresponding to the saturation binding data in panel A) and mock-transfected membranes (open circles).

Table 2
K_D and B_{max} values for binding of [^{3}H]A-778317 to rat DRG and spinal cord membranes, and comparison to rTRPV1 (transient transfection) membranes.

Membranes	K_D (nM)	B_{max} (pmol/mg protein)
rTRPV1 (transient transfection)	8.17 ± 0.37	21.8 ± 0.5
Rat DRG	9.97 ± 2.10	3.33 ± 0.23
Rat spinal cord	8.37 ± 1.81	0.35 ± 0.05

Data are shown as means ± S.E.M. for three determinations.

resiniferatoxin (K_i = 25.8 nM), and like resiniferatoxin, it partially inhibited the specific binding of the radioligand. In contrast, olvanil was much less potent than resiniferatoxin and tinyatoxin (K_i = 2.1 μM), but completely inhibited the specific binding of the radioligand. Capsaicin was also a weak inhibitor of the binding (K_i = 11.1 μM). Of the TRPV1 receptor antagonists tested, A-778317 was the most potent (K_i = 28.0 nM), and it was approximately 7-fold more potent than its weaker enantiomer A-778316 (K_i = 203 nM) (Fig. 4B, Table 3). Rank order potency of the antagonists was A-778317 > A-778316 > A-425619 = A-784168 > capsazepine. Furthermore, each of the antagonists completely inhibited the specific binding of the radioligand, with Hill slopes close to 1.

3.3. Binding of [^{3}H]A-778317 to native TRPV1 channels in rat DRG

Saturable high-affinity binding of [^{3}H]A-778317 was detected in rat DRG membranes (Fig. 5A). The apparent K_D of the radioligand, derived from the saturation binding data, was 9.97 ± 2.10 nM (n = 3), and the B_{max} was 3.33 ± 0.23 pmol/mg protein (n = 3) (Table 2). The K_D of the radioligand for the native TRPV1 channels in rat DRG was approximately the same as its K_D for the recombinant rTRPV1 receptor. Also, as observed for rTRPV1 (transient transfection) membranes, a monophasic Scatchard plot of the saturation binding

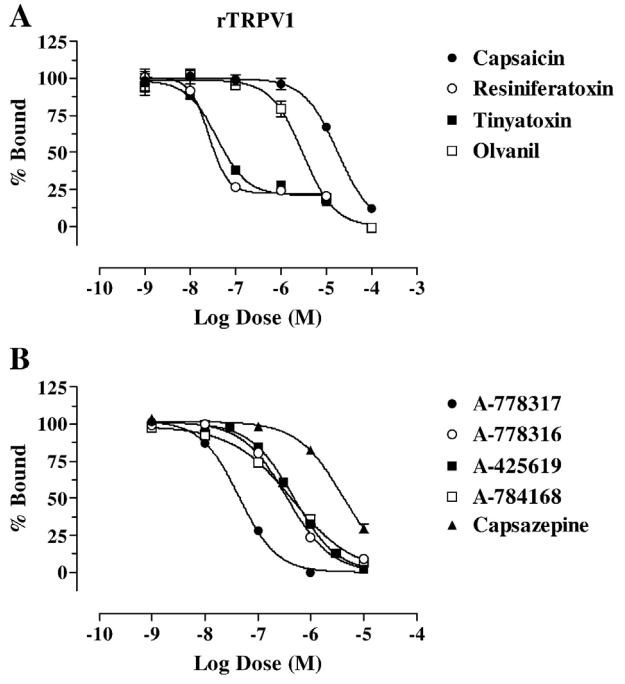

Fig. 4. Competition for [^{3}H]A-778317 binding to rTRPV1 (transient transfection) membranes. Specific binding of [^{3}H]A-778317 was reversible, and inhibited by both TRPV1 receptor agonists and antagonists. A, Concentration–inhibition curves of TRPV1 receptor agonists; and B, Concentration–inhibition curves of TRPV1 receptor antagonists, in the presence of 5 nM radioligand. Data are expressed as a percentage of the specific bound counts (% bound) observed in the absence of test compound, and represent the means ± S.E.M. for three or more determinations.

Table 3
Potencies of TRPV1 agonists and antagonists in a binding assay for the recombinant rTRPV1 receptor.

Compound	Inhibition of 5 nM [^{3}H]A-778317 binding: rTRPV1 (transient transfection) membranes	
Agonists	K_i (nM)	n_H
Resiniferatoxin	17.5 ± 2.2	2.24 ± 0.29
Tinyatoxin	25.8 ± 4.2	1.56 ± 0.34
Olvanil	2130 ± 360	1.24 ± 0.24
Capsaicin	11,100 ± 640	1.12 ± 0.08
Antagonists	K_i (nM)	n_H
A-778317	28.0 ± 5.0	1.24 ± 0.12
A-778316	203 ± 11	1.15 ± 0.21
A-425619	298 ± 24	1.06 ± 0.05
A-784168	299 ± 66	0.75 ± 0.09
Capsazepine	2640 ± 385	1.01 ± 0.05

Data are shown as means ± S.E.M. for three or more determinations.

was obtained with rat DRG membranes (Fig. 5C). Nonspecific binding of 5 nM [^{3}H]A-778317 comprised about 30% of the total bound counts.

The potencies of TRPV1 receptor agonists and antagonists to inhibit the specific binding of 5 nM [^{3}H]A-778317 were generally lower in rat DRG membranes than rTRPV1 (transient transfection) membranes. Capsaicin was slightly less potent at the native rTRPV1 receptor (26.9% inhibition at 10 μM) (Fig. 6A, Table 4) than the recombinant rTRPV1 receptor. However, resiniferatoxin, tinyatoxin and olvanil were much less potent at the native rTRPV1 receptor (resiniferatoxin: 21.5% inhibition at 10 μM; tinyatoxin: 34.2% inhibition at 10 μM; and olvanil: 11.7% inhibition at 10 μM). Of the TRPV1 receptor antagonists tested, A-778317 was the most potent (K_i = 28.3 nM), and it was followed in order of potency by A-778316 (K_i = 482 nM) and A-425619 (K_i = 752 nM) (Fig. 6B, Table 4). Both A-778317 and A-425619 completely inhibited the specific binding of the radioligand (at 1 and 10 μM, respectively), with Hill slopes close to 1. A-778316 inhibited the specific binding by 81% at 3 μM. A-784168 was found to be a weak inhibitor of binding (50.7% inhibition at 10 μM), less potent than A-425619 and approximately equipotent to capsazepine.

3.4. Binding of [^{3}H]A-778317 to native TRPV1 channels in rat spinal cord

The binding of [^{3}H]A-778317 to the native TRPV1 channels in rat spinal cord resembled its binding to the native TRPV1 channels in rat DRG. Similar to rat DRG membranes, saturable high-affinity binding of [^{3}H]A-778317 was detected in rat spinal cord membranes (Fig. 5B). The apparent K_D of the radioligand, derived from the saturation binding data, was 8.37 ± 1.81 nM (n = 3), and the B_{max} was 0.352 ± 0.049 pmol/mg protein (n = 3) (Table 2). The K_D values for binding of the radioligand to the native TRPV1 channels in rat DRG and spinal cord were approximately the same. However, the number of binding sites per milligram protein was about 10-fold lower in rat spinal cord membranes than DRG membranes. A Scatchard plot of the saturation binding data remained monophasic (Fig. 5D). Nonspecific binding of 5 nM [^{3}H]A-778317 to rat spinal cord membranes was higher than rat DRG membranes and comprised about 60% of the total bound counts. The same amount of nonspecific binding was observed whether membranes were prepared from frozen or freshly dissected rat spinal cord.

Ligand-competition for the binding of [^{3}H]A-778317 was similar to the native TRPV1 channels in rat DRG. All the TRPV1 receptor agonists tested were found to be weak inhibitors of the binding (capsaicin: 49.3% inhibition at 10 μM; resiniferatoxin: 19.2% inhibition at 10 μM; tinyatoxin: 28.6% inhibition at 10 μM; and olvanil: 48.5% inhibition at 10 μM) (Table 4). Of the TRPV1 receptor antagonists tested, A-778317 was the

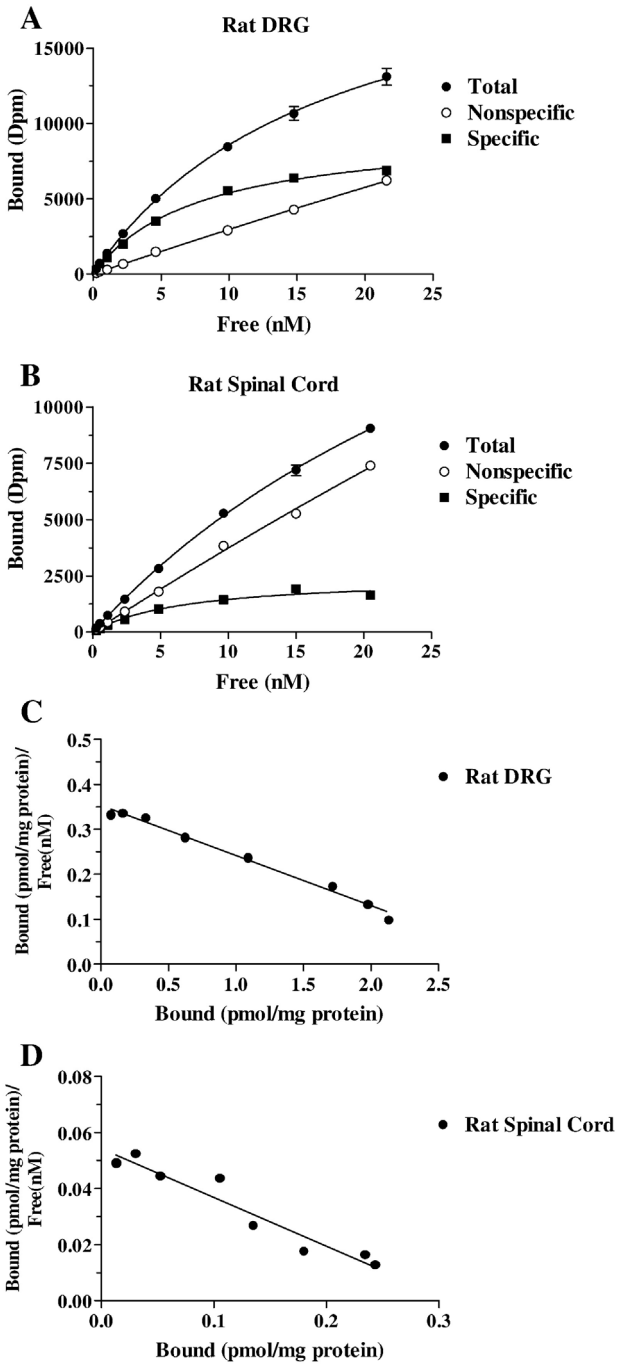

Fig. 5. Saturation binding of [^{3}H]A-778317 to rat DRG and spinal cord membranes. The amount of membrane protein in the binding assay was 50 μg, for rat DRG membranes; and 100 μg, for rat spinal cord membranes. A and B, Representative saturation binding curves for rat DRG and spinal cord membranes, respectively. Total bound counts (dpm) (filled circles) and nonspecific bound counts (dpm) (open circles) are shown for eight different radioligand concentrations. Specific bound counts (dpm) (filled squares) were calculated by subtracting nonspecific from total bound counts. Specific binding of [^{3}H] A-778317 was saturable. The number of binding sites was 10-fold higher in rat DRG membranes than rat spinal cord membranes. Mean B_{max} of the specific binding was 3.33 ± 0.23 and 0.35 ± 0.05 pmol/mg protein ($n = 3$), respectively, for rat DRG and spinal cord membranes. C and D, Corresponding Scatchard plots of the specific binding data.

most potent ($K_i = 51.1$ nM), and it was followed in order of potency by A-425619 ($K_i = 742$ nM), A-778316 ($K_i = 902$ nM), A-784168 (70.7% inhibition at 10 μM) and capsazepine (42.4% inhibition at 10 μM)

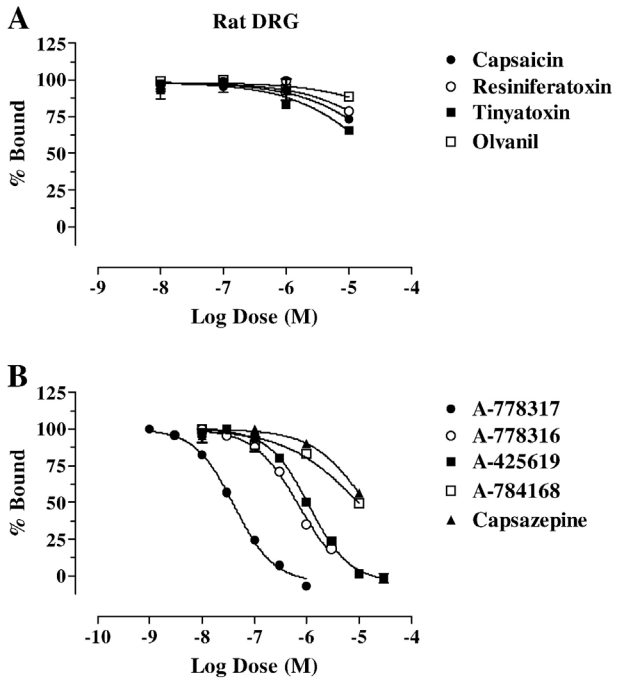

Fig. 6. Competition for [^{3}H]A-778317 binding to rat DRG membranes. Specific binding of [^{3}H]A-778317 was inhibited by both TRPV1 receptor agonists and antagonists. However, contrary to their effects at the recombinant rTRPV1 receptor, the TRPV1 agonists resiniferatoxin and tinyatoxin inhibited the binding of [^{3}H]A-778317 to rat DRG membranes weakly (<50% inhibition at 10 μM). A, Concentration–inhibition curves of TRPV1 receptor agonists; and B, Concentration–inhibition curves of TRPV1 receptor antagonists, in the presence of 5 nM radioligand. Data are expressed as a percentage of the specific bound counts (% bound) observed in the absence of test compound, and represent the means ± S.E.M. for three or more determinations.

(Table 4). Both A-778317 and A-425619 completely inhibited the specific binding, with Hill slopes close to 1.

4. Discussion

The TRPV1 protein has six transmembrane (TM) domains, intracellular N- and C-termini, and a pore loop between the fifth and sixth TM domains. It coassembles with three other identical or homologous subunits to form a tetrameric ion channel complex. Key amino acid residues have been identified near the TM3 region of the

Table 4
Potencies of TRPV1 agonists and antagonists in a binding assay for native rTRPV1 channels.

Compound	Inhibition of 5 nM [^{3}H]A-778317 binding:			
	Rat DRG membranes		Rat spinal cord membranes	
Agonists	% inhibition at 10 μM		% inhibition at 10 μM	
Resiniferatoxin	21.5 ± 2.3		19.2 ± 4.5	
Tinyatoxin	34.2 ± 2.8		28.6 ± 5.0	
Capsaicin	26.9 ± 2.8		49.3 ± 3.0	
Olvanil	11.7 ± 3.4		48.5 ± 3.9	
Antagonists	K_i (nM)	n_H	K_i (nM)	n_H
A-778317	28.3 ± 2.4	1.09 ± 0.04	51.1 ± 7.7	0.94 ± 0.07
A-778316	482 ± 28	1.05 ± 0.03	902 ± 119	0.86 ± 0.07
A-425619	752 ± 45	1.18 ± 0.04	742 ± 216	0.99 ± 0.09
	% inhibition at 10 μM		% inhibition at 10 μM	
A-784168	50.7 ± 2.9		70.7 ± 5.8	
Capsazepine	44.0 ± 3.1		42.4 ± 3.8	

Data are shown as means ± S.E.M. for three or more determinations.

TRPV1 protein (Tyr^{511} and Ser^{512}) that may constitute a portion of the capsaicin binding site (Jordt and Julius, 2002), while key molecular determinants for proton activation (Glu^{600} and Glu^{648}) have been identified in the pre-pore loop (Jordt et al., 2000). It is believed that protons and heat induce an allosteric change in the four assembled TRPV1 subunits that is sufficient to gate the channel (Latorre et al., 2007; Ryu et al., 2007). Subthreshold levels of protons and heat may also act as modulators of TRPV1 receptor function, and sensitize the TRPV1 channel to activation by endogenous ligands. It is known that both protonation and phosphorylation of the TRPV1 protein can sensitize the functional channel to activation by endogenous ligands, and also heat (Jordt et al., 2000; Julius and Basbaum, 2001; Vellani et al., 2001; Bianchi et al., 2006).

The binding properties of [^{3}H]A-778317 at the recombinant rTRPV1 receptor were ascertained in rTRPV1 (transient transfection) membranes. The apparent K_D of [^{3}H]A-778317 for the recombinant rTRPV1 receptor, as derived from saturation binding data, was determined to be 8.17 nM, which is higher than its K_D for the recombinant hTRPV1 receptor (apparent $K_D = 3.39$ nM) (Bianchi et al., 2007a). Its B_{max} was 21.8 pmol/mg protein. A Scatchard plot of the saturation binding data was monophasic, indicating that [^{3}H]A-778317 labeled a single class of high-affinity binding sites. Although some high-affinity binding was also detected in mock-transfected membranes, it represented a small percentage (about 10%) of the specific bound counts in rTRPV1 (transient transfection) membranes.

The binding of [^{3}H]A-778317 to the recombinant rTRPV1 receptor was inhibited in a dose-dependent manner by both TRPV1 agonists and antagonists. Rank order potencies of the TRPV1 agonists and antagonists correlated well with their rank order potencies in a Ca^{2+} influx assay for the recombinant rTRPV1 receptor, with one notable exception, *i.e.* capsaicin. Whereas capsaicin was a potent agonist in the Ca^{2+} influx assay ($EC_{50} = 8.4$ nM), it was a weak inhibitor in the binding assay ($K_i = 11.1$ μM). Capsaicin has also been reported to be a weak inhibitor in other binding assays that use [^{3}H]resiniferatoxin (Szallasi et al., 1999) and [^{125}I]iodo-resiniferatoxin (Wahl et al., 2001) to label the recombinant rTRPV1 receptor. The binding of [^{3}H]A-778317 to the recombinant rTRPV1 receptor was completely inhibited by capsaicin, and also by olvanil ($K_i = 2.13$ μM), which is a TRPV1 receptor agonist structurally related to capsaicin. Resiniferatoxin, and the resiniferatoxin analogue tinyatoxin, were much more potent ($K_i = 17.5$ and 25.8 nM, respectively) than capsaicin in the binding assay, and their binding potencies were nearer to their functional potencies in the Ca^{2+} influx assay ($EC_{50} = 4.1$ and 4.2 nM, respectively). However, resiniferatoxin and tinyatoxin did not fully inhibit the binding of [^{3}H]A-778317 to the recombinant rTRPV1 receptor. The reasons for these differences are unclear, but could be related to the binding sites of the agonists, their extent of overlap with the binding site for A-778317, the interaction of the agonists with key amino acid residues within the binding space, and agonist-induced changes in conformation of the TRPV1 protein.

The location of the binding site for A-778317 is not known with certainty. A pharmacophore model of the TRPV1 channel (Kym et al., 2009) suggests that certain TRPV1 receptor antagonist pharmacophores have a common binding site located within the pore region of the tetrameric channel. This may apply to A-778317 as well. The antagonist pharmacophore is proposed to form critical hydrogen bond interactions with Tyr^{667} residues on opposing monomers of the ion channel complex. The extent of overlap between this common antagonist binding site and the binding sites for capsaicin, resiniferatoxin and other TRPV1 receptor agonists is not clear. Mutagenesis studies suggest that the binding site for capsaicin is located near the third intracellular loop and TM3 region of the TRPV1 protein (Jordt and Julius, 2002; Gavva et al., 2004). It is believed that resiniferatoxin binds to the same site as capsaicin, but interacts with key amino acid residues within the binding space differently (Chou et al., 2004). Schild analyses indicated that A-778317 is a competitive antagonist of capsaicin and resiniferatoxin at the recombinant rTRPV1 receptor. The concentration–effect curves of capsaicin and resiniferatoxin were both shifted to the right in the presence of A-778317 without loss of efficacy. More importantly, it was found that the linear regression curve-fit of the Schild plot data (log (dose ratio − 1) versus the log [A-778317] (M)) did not differ between capsaicin and resiniferatoxin, giving nearly identical slopes and X-intercepts. These data support the idea that capsaicin and resiniferatoxin gate the TRPV1 channel by binding to the same site (or sites) within the ion channel complex.

The aim of the present study was to determine whether [^{3}H]A-778317 could be used to label native TRPV1 channels in rat tissues. High-affinity binding of [^{3}H]A-778317 was in fact detected in membrane preparations of both rat DRG and spinal cord. The binding was saturable and reversed by unlabelled A-778317, its weaker enantiomer A-778316 and A-425619. [^{3}H]A-778317 appeared to label a single class of binding sites in rat DRG and spinal cord membranes with similar affinity (apparent $K_D = 10$ and 8.4 nM, respectively). The affinity of [^{3}H]A-778317 for these binding sites was about the same as that observed for the recombinant rTRPV1 receptor. The number of binding sites was approximately 10-fold higher in rat DRG membranes than rat spinal cord membranes ($B_{max} = 3.3$ and 0.35 pmol/mg protein, respectively). Specificity of [^{3}H]A-778317 for the native TRPV1 channels was low in spinal cord membranes, but comparable to that reported for [^{125}I]iodo-resiniferatoxin (Wahl et al., 2001). The nonspecific binding of 5 nM [^{3}H]A-778317 comprised a higher percentage of the total bound counts in rat DRG and spinal cord membranes (30% and 60%, respectively) than rTRPV1 (transient transfection) membranes (10%). Despite these percentage differences, the actual nonspecific bound counts per milligram protein were roughly the same. Since A-778317 is a lipophilic compound, most of the nonspecific binding is likely due to partitioning of the radioligand within the lipid bilayer of the membranes.

The pharmacology of the high-affinity binding was characterized in rat DRG and spinal cord membranes, and compared to the pharmacology of the recombinant rat TRPV1 receptor, in order to determine whether binding of [^{3}H]A-778317 is occurring to the native rTRPV1 channels. It was discovered that the pharmacology of the high-affinity binding sites in rat DRG and spinal cord membranes was distinct from the recombinant rTRPV1 receptor. The potencies of TRPV1 receptor agonists were generally lower in rat DRG and spinal cord membranes than rTRPV1 (transient transfection) membranes. Resiniferatoxin and tinyatoxin showed the biggest disparity in potencies, inhibiting the binding of [^{3}H]A-778317 to rat DRG and spinal cord membranes weakly (about 20–30% inhibition at 10 μM), whereas they were potent inhibitors of binding to rTRPV1 (transient transfection) membranes ($K_i = 17.5$ and 25.8 nM, respectively). This represents a greater than 300-fold difference in potency for these compounds between native and recombinant rTRPV1 receptors. Cell-specific modulation of the TRPV1 channel may account for the difference observed between native and recombinant rTRPV1 receptors. The factors/pathways that regulate the native TRPV1 channels in rat DRG and spinal cord are likely different from the ones present in a clonal cell line. Changes in conformation of the ion channel complex could alter the binding site for [^{3}H]A-778317, and also modify the overlap/connection between this binding site and nearby agonist binding sites. The potencies of TRPV1 receptor antagonists, with the exception of A-778317 itself, were also lower in rat DRG and spinal cord membranes than rTRPV1 (transient transfection) membranes. A large disparity in potency was evident for A-784168. Whereas A-784168 inhibited the binding of [^{3}H]A-778317 to DRG and spinal cord membranes weakly (50.7% and 70.7% inhibition at 10 μM, respectively), it was a strong inhibitor of binding to rTRPV1 (transient transfection) membranes and showed the same potency as A-425619 ($K_i = 299$ and 298 nM, respectively). It is uncertain why the potency of A-784168 was shifted more than A-425619 and A-778316, but could be related to its chemotype. A-425619 and A-778316 are both structurally related to

A-778317 (isoquinolinyl-ureas), whereas A-784168 is an optimized ligand from a different chemical (tetrahydropyridine-carboxamide) series. The interaction with key amino acid residues within the antagonist binding space may subtly differ between chemotypes, and also could be differentially affected by changes in conformation of the ion channel complex.

The pharmacology of native TRPV1 channels may vary depending on their location, their interaction with surrounding lipids and proteins present in the membranes, accessibility to TRPV1 ligands, and presence of cell-specific regulatory factors/pathways. Indeed, the structure and function of many ion channels are modulated by the mutable properties of the membranes in which they reside (Tillman and Cascio, 2003). In the case of TRPV1, it is known that various endogenous lipids can act either as allosteric modulators or direct activators of the receptor. These endogenous lipids include the endocannabinoid anandamide, long-chain unsaturated N-acyl dopamines, typified by NADA and N-oleoyldopamine (Huang et al., 2002; Chu et al., 2003), 12- and 15-(S)-hydroperoxyeicosatetraenoic acids (Hwang et al., 2000), oleoylethanolamide and other N-acylethanolamines (Ahern, 2003; Movahed et al., 2005; Wang et al., 2005), omega-3 polyunsaturated fatty acids (Matta et al., 2007) and cholesterol (Liu et al., 2006). Various endogenous proteins, i.e., splice variants of TRPV1, are also known to negatively regulate TRPV1 channel activity.

Several N-terminal splice variants of the TRPV1 receptor have been identified, including stretch-inactivated channel (Suzuki et al., 1999), vanilloid receptor 5′ splice variant (VR.5′sv)(Schumacher et al., 2000; Xue et al., 2001), VR1L2 (Xue et al., 2001) and TRPV1b (Lu et al., 2005, Vos et al., 2006). The VR.5′sv splice variant has in fact been found to be present in rat DRG, spinal cord and brain (Sanchez et al., 2001). The VR.5′sv homomultimer is nonfunctional, but co-assembly of VR.5′sv with TRPV1 can result in the formation of heteromultimers with functional properties intermediate between TRPV1 and VR.5′sv. Depending on their level of co-expression with the full-length TRPV1 protein, it is possible that splice variants could contribute to the high-affinity binding of [^{3}H]A-778317 in rat DRG and spinal cord membranes, and thereby alter the pharmacology. Similarly, heteromultimers of TRPV1 and other vanilloid receptor proteins could also contribute to the overall high-affinity binding. It has been reported that heteromultimer TRPV1/TRPV2 channel complexes can form in rat DRG (Liapi and Wood, 2005; Rutter et al., 2005) and cerebral cortex (Liapi and Wood, 2005). Thus, the binding of [^{3}H]A-778317 in rat tissues could be complex and involve not only functional homomultimeric TRPV1 ion channels, but also heteromultimeric ion channel complexes that exhibit distinctive binding and functional properties of their own.

In summary, we performed studies to determine whether [^{3}H]A-778317 can be used to label native TRPV1 channels in rat tissues. Saturable high-affinity binding of [^{3}H]A-778317 was detected in both rat DRG and spinal cord membranes. Furthermore, in both rat DRG and spinal cord membranes, [^{3}H]A-778317 bound to a single class of high-affinity binding sites. It was discovered, however, that the pharmacology of the high-affinity binding sites in rat DRG and spinal cord was distinct from the recombinant rTRPV1 receptor. This raises a concern that the binding of [^{3}H]A-778317 to native TRPV1 channels in rat tissues could be complex. Additional studies are necessary to establish the selectivity of A-778317 for the functional homomultimeric TRPV1 ion channel over heteromultimeric ion channel complexes that are co-expressed in the same tissues. [^{3}H]A-778317 may prove to be a useful radioligand to study changes in the pharmacology of native TRPV1 channels under pathophysiological conditions.

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