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Proton activation does not alter antagonist interaction with the capsaicin-binding pocket of TRPV1

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Running Title: Proton gating of TRPV1 does not alter antagonist affinities

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Abbreviations: TRPV1, transient receptor potential vanilloid type 1; AMG0610, (2E)-3-(6-*tert*-butyl-2-methylpyridin-3-yl)-N-(1H-indol-6-yl)acrylamide; AMG6880, (2E)-3-[2-piperidin-1-yl-6-(trifluoromethyl)pyridin-3-yl]-N-quinolin-7-ylacrylamide; AMG7472, 5-chloro-6-{(3R)-3-methyl-4-[6-(trifluoromethyl)-4-(3,4,5-trifluorophenyl)-1H-benzimidazol-2-yl]piperazin-1-yl}pyridin-3-yl)methanol; AMG9810, (*E*)-3-(4-*t*-butylphenyl)-*N*-(2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)acrylamide; A-425619, (1-Isoquinolin-5-yl-3(4-trifluoromethyl-benzyl)-urea; BCTC, N-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine -1(2H)-carboxamide; JNJ-17203212, 4-(3-trifluoromethylpyridin-2-yl)piperazine-1-carboxylic acid (5-trifluoromethylpyridin-2-yl)amide; SB-366791, (2E)-3-(4-chlorophenyl)-N-(3-methoxyphenyl)acrylamide; TM3/4, transmembrane domains 3 and 4

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Abstract

Vanilloid receptor 1 (TRPV1) is activated by chemical ligands (e.g., capsaicin and protons) and heat. Here we show that AMG6880, AMG7472, and BCTC are potent antagonists of rat TRPV1 activation by either capsaicin or protons (pH 5) (defined here as group A antagonists), whereas AMG0610, capsazepine, and SB-366791 are antagonists of capsaicin, but not proton, activation (defined here as group B antagonists). By utilizing capsaicin-sensitive and insensitive rabbit TRPV1 channels, we show that antagonists require the same critical molecular determinants located in the TM3/4 region to block both capsaicin and proton activation, suggesting the presence of a single binding pocket. To determine if the differential pharmacology is a result of proton activation-induced conformational changes in the capsaicin-binding pocket that alter group B antagonist affinities, we have developed a functional antagonist competition assay. We hypothesized that if group B antagonists bind at the same or an overlapping binding pocket of TRPV1 as group A antagonists, and proton activation does not alter the binding pocket, then group B antagonists should compete with and prevent group A antagonism of TRPV1 activation by protons. Indeed, we found that each of the group B antagonists competed with and prevented BCTC, AMG6880 or AMG7472 antagonism of rat TRPV1 activation by protons with pA_2 values similar to those for blocking capsaicin, indicating that proton activation does not alter the conformation of the TRPV1 capsaicin-binding pocket. In conclusion, group A antagonists appear to lock the channel conformation in the closed state, blocking both capsaicin and proton activation.

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Introduction

Vanilloid Receptor 1 (*a.k.a.* VR1 and TRPV1: Caterina, et al., 1997, Montell et al., 2002) has been cloned and shown to be a non-selective cation channel with high permeability to calcium. TRPV1 can be activated by chemical ligands (capsaicin, resiniferatoxin, anandamide, 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid, *N*-arachidonyl dopamine, *N*-oleoyldopamine and protons [pH $\leq$ 5.7]), and by physical stimuli, such as heat (>42 ^{o}C) acting as an integrator of multiple noxious stimuli (Holzer, 2004 and references therein; Szolcsanyi J. 2004 and references therein; Tominaga et al., 1998). TRPV1 is abundantly expressed in peripheral sensory neurons and is thought to contribute to increased nociceptor function in pain states (Holzer, 2004; Ji et al., 2002; Julius and Basbaum, 2001; Szallasi & Blumberg 1999). TRPV1 expression is increased after inflammatory injury in rodents, and the increased level of TRPV1 protein combined with the confluence of stimuli present in inflammatory injury states has been proposed to result in a reduced threshold of activation of nociceptors that express TRPV1, i.e., hyperalgesia (Ji et al., 2002). In agreement with this finding, TRPV1 knockout mice display reduced thermal hypersensitivity following inflammatory tissue injury (Caterina et al., 2000; Davis et al., 2000).

Recently, several competitive antagonists of TRPV1 have been reported, which prevent activation by different stimuli (reviewed in Szallasi and Appendino, 2004), such as BCTC (Valenzano et al., 2003), SB-366791 (Gunthorpe et al., 2004), AMG9810 (Gavva et al., 2005), AMG6880 (compound 49b in Doherty et al., 2005), JNJ-17203212 (Ghilardi et al., 2005) and A-425619 (Honore et al., 2005). Among the reported TRPV1 antagonists, AMG9810, A-425619, and BCTC inhibit hyperalgesia in models of inflammatory pain (Gavva et al., 2005; Honore et al., 2005; Pomonis et al., 2003), A-425619 reverses skin incision-induced thermal hyperalgesia (Honore et al., 2005), and JNJ-17203212 attenuates bone cancer pain (Ghilardi et al., 2005) supporting a role for TRPV1 in clinical pain states.

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Among the reported TRPV1 antagonists, some compounds (defined here as group A antagonists) block both capsaicin and proton activation (AMG6880, AMG9810, and BCTC), whereas others only block capsaicin, but not proton activation (defined here as group B antagonists), in a species-dependent manner. For example, capsazepine blocks the proton activation of human and guinea pig, but not rat TRPV1 (McIntyre et al., 2001; Savidge et al., 2001). We have shown that SB-366791 blocks capsaicin, but not proton (pH 5) activation of rat TRPV1 (Gavva et al., 2005). These findings raise the question of whether the differential pharmacology of group A versus group B antagonists is a result of proton activation-induced conformational changes in the capsaicin-binding pocket of rat TRPV1 that alter antagonist affinities or if it is because they act through different binding sites on TRPV1 to block capsaicin and proton activation.

Interestingly, the ability of antagonists to block all modes of activation of TRPV1 appears to correlate with their *in vivo* efficacy in animal models of pain (Walker et al., 2003; Gavva et al., 2005; Honore et al., 2005; Pomonis et al., 2003). For example, capsazepine produced significant anti-hyperalgesic effects in a model of inflammatory and nerve-injury related pain in guinea pigs, but not in rats, correlating with its ability to block all modes of activation of guinea pig TRPV1 (McIntyre et al., 2001; Savidge et al., 2001; Walker et al., 2003). Similarly, AMG9810, A-425619, and BCTC, all of which block capsaicin, proton and heat activation of rat TRPV1, were also effective as anti-hyperalgesics in rat models of inflammatory pain (Gavva et al., 2005; Honore et al., 2005; Pomonis et al., 2003). Hence we are interested in understanding the differential pharmacology of antagonists and their mechanisms of action for blocking capsaicin and proton activation.

The aim of our study was to investigate whether the differential pharmacology of group A and B antagonists is due to proton activation-induced conformational changes in the capsaicin-binding pocket of TRPV1 that alter antagonist affinities. Normally this question would be addressed with radioligand binding experiments. However, tritiated-resiniferatoxin is the only reported ligand that can be used in a competition-binding assay for TRPV1, and the assay is not optimal at pH 5 (Szallasi and Blumberg, 1993).

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Furthermore, the critical molecular determinants for resiniferatoxin affinity in binding assays and resiniferatoxin/capsaicin agonism (efficacy) in functional assays are also known to differ in TRPV1 (Acs et al., 1996, Gavva et al., 2004). Here, by utilizing a functional cell-based antagonist competition assay, we show that group B antagonists (AMG0610, capsazepine, and SB-366791) compete with each of the group A antagonists (AMG7472, AMG6880, and BCTC), indicating that proton activation does not alter the capsaicin-binding pocket or the antagonist affinities.

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Methods

Cloning and stable transfections. Cloning and stable cell generation for rat and rabbit TRPV1 (and its mutants) were described in Gavva et al., (2004). Chinese hamster ovary (CHO) cells stably expressing TRPV1 were maintained in DMEM supplemented with 10% dialyzed FBS, 800 µg/ml Genetecin, penicillin, streptomycin, L-glutamine, and non-essential amino acids. Functional TRPV1 channels are present at the plasma membrane, as well as at the intracellular membranes, such as endoplasmic reticulum (Karai et al., 2004). Since we have utilized agonist-induced $^{45}Ca^{2+}$ uptake as the readout, it should be noted that the pharmacology of agonists and antagonists reported in this paper is reflecting the activity of the plasma membrane TRPV1 channels.

$^{45}Ca^{2+}$ uptake assay. Two days prior to the assay, cells were seeded in Cytostar 96 well plates (Amersham) at a density of 20,000 cells/well. The activation of TRPV1 is followed as a function of cellular uptake of radioactive calcium ($^{45}Ca^{2+}$, ICN). All the $^{45}Ca^{2+}$ uptake assays had a final $^{45}Ca^{2+}$ concentration at 10 µCi/mL.

Capsaicin Antagonist Assay. Compounds were pre-incubated with TRPV1 expressing CHO cells in HBSS (Hank's buffered saline solution) supplemented with BSA 0.1mg/ml and 1 mM HEPES at pH 7.4 at room temperature for 2 minutes prior to addition of $^{45}Ca^{2+}$ and capsaicin (final concentration, 0.5 µM) in F12 media and then left for an additional 2 minutes prior to compound washout.

Proton Antagonist Assay. Compounds were pre-incubated with CHO cells expressing TRPV1 at room temperature for 2 minutes prior to addition of $^{45}Ca^{2+}$ in F12 media supplemented with 30 mM HEPES, 30mM MES and 0.1mg/ml BSA adjusted to pH 4.1 with HCl (final assay pH 5) and then left for an additional 2 minutes prior to compound washout.

Antagonist competition assay. CHO cells expressing rat TRPV1 were incubated with antagonist mixtures (for example: 0, 1.1, 3.3 or 10 µM [final concentration] capsazepine

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combined with each of the 400, 133, 44.3, 14.7, 4.9, 1.65, 0.51, 0.17, 0.056, and 0.014 nM [final concentration] BCTC) for 5 min before the addition of $^{45}Ca^{2+}$ in F12 media supplemented with 30 mM Hepes, 30mM MES and 0.1mg/ml BSA adjusted to pH 4.1 with HCl (final assay pH 5) and then left for an additional 2 minutes prior to compound washout.

Compound Washout and Analysis. Assay plates were washed 2 times with PBS, 0.1 mg/mL BSA using an ELX405 plate washer (Bio-Tek Instruments Inc.) immediately after the functional assay. Radioactivity in the 96 well plates was measured using a MicroBeta Jet (Perkin-Elmer Inc.). IC_{50} and pA_2 values were calculated by generating antagonist competition curves and Schild plots using Graphpad Prism 4.01 (GraphPad Software Inc, San Diego, CA).

Reagents. All the cell culture reagents were purchased from Invitrogen (Carlsbad, CA). AMG0610 (Bo et al., 2003), AMG7472 (Balan et al., 2004), and BCTC were synthesized at Amgen Inc, Thousand Oaks, CA (>95% purity, NMR, Mass, and CHN analysis confirmed). Other reagents were obtained from the following companies: capsaicin (cat # 211274), Calbiochem, Sand Diego, CA, capsazepine (cat # 0464), SB-366791 (cat # 1615), Tocris Cookson Inc, Ellisville, MO, SB-366791 (cat # S-0441), Sigma Inc, St. Louis, MO. Structures of antagonists used in this study are shown in Figure 1A.

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Results

Characterization of TRPV1 antagonists in agonist-induced $^{45}Ca^{2+}$ uptake assay. AMG7472, a novel benzimidazole (Balan et al., 2004), is identified as an antagonist of both capsaicin and proton (pH 5) activation of TRPV1, whereas AMG0610 is identified as an antagonist of capsaicin, but not proton activation of rat TRPV1 (Fig.1B,C). As reported previously, BCTC (Valenzano et al., 2003) and AMG6880 (compound 49b in Doherty et al., 2005) blocked both capsaicin and proton activation, whereas capsazepine (McIntyre et al., 2001) and SB-366791 (Gavva et al., 2005) blocked only capsaicin but not proton activation of rat TRPV1 (Fig.1B,C). In fact, at higher concentrations (> 0.3 μM), AMG0610 and SB-366791 showed a concentration-dependent potentiation of pH 5-induced $^{45}Ca^{2+}$ uptake in CHO cells expressing rat TRPV1 but not in untransfected cells (Fig. 1C and data not shown). We have defined the compounds that block both capsaicin and proton activation of rat TRPV1 as 'group A antagonists' (AMG6880, AMG7472, and BCTC) and those that only block capsaicin, but not proton activation as 'group B antagonists' (AMG0610, capsazepine, and SB-366791) in this manuscript.

To determine if AMG0610, AMG6880, AMG7472, BCTC, capsazepine, and SB-366791 are competitive antagonists at rat TRPV1, we tested concentration-response curves for the induction of $^{45}Ca^{2+}$ uptake by capsaicin, as a function of the concentration of above TRPV1 antagonists. For all the antagonists, concentration-response curves for induction of $^{45}Ca^{2+}$ uptake in CHO cells expressing rat TRPV1 by capsaicin showed parallel rightward shifts with no depression of the maximum response, indicating competitive antagonism (Fig. 1D, E and data not shown). The pA_2 values determined by Schild analysis are shown in Table 1. The rank order of potency against capsaicin activation is BCTC $\geq$ AMG7472 > AMG6880 > AMG0610 > capsazepine $\geq$ SB-366791.

Molecular determinants required for antagonist action are present in the transmembrane domains 3 and 4 (TM3/4) region of TRPV1. Our data and that of other investigators have previously shown that agonists such as capsaicin, resiniferatoxin, endogenous ligands, as well as antagonists like BCTC, and capsazepine require the

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TM3/4 region and Thr^{550} for their actions at TRPV1 (Chou et al., 2004; Gavva et al., 2004; Jordt and Julius, 2002; Phillips et al., 2004). Since several antagonists used in this study represent novel chemical scaffolds and show differential pharmacology, we have tested their activity at the capsaicin-insensitive rabbit TRPV1 (Gavva et al., 2004) and the capsaicin-sensitive rabbit TRPV1 mutant (I550T) to determine if the same critical determinants in the TM3/4 region are required for antagonist action. Protons (pH 5) activate both rabbit TRPV1 and rabbit TRPV1-I550T equivalently. We hypothesized that if group A antagonists act through separate sites to block capsaicin and proton activation, and group B antagonists only bind at one site to block capsaicin activation and are unable to bind at a second site that is required for blocking proton activation, then group A, but not group B, antagonists should block proton activation of the capsaicin-insensitive rabbit TRPV1.

All the antagonists tested are ineffective (IC_{50} > 4000 nM) against blocking proton activation of capsaicin-insensitive rabbit TRPV1 (Table 2). AMG6880, AMG7472, BCTC, and capsazepine blocked both proton and capsaicin activation of the capsaicin-sensitive rabbit TRPV1-I550T mutant, confirming that Thr^{550} is a critical determinant for antagonist action (Table 2). Capsaicin activation of rabbit TRPV1-I550T was blocked by all TRPV1 antagonists used in this study, but not by a $P2X_{2/3}$ antagonist, A-317491 (Jarvis et al., 2002; Table 2) further indicating that all the TRPV1 antagonists require the same critical determinants for blocking both capsaicin and proton activation. The findings that, i) group A antagonists tested are ineffective against blocking proton activation of capsaicin-insensitive rabbit TRPV1 (Table 2), ii) group B antagonists neither blocked nor potentiated pH 5 activation of rabbit TRPV1, and iii) both group A and B are antagonists of capsaicin activation of rabbit TRPV1-I550T indicate that all of these antagonists act through the same or an overlapping binding pocket to block both capsaicin and proton activation. The fact that protons activate rabbit TRPV1 and that group A antagonists did not block proton activation of rabbit TRPV1, a channel that lacks an optimal capsaicin-binding pocket, suggests that group A antagonists are non-competitive with respect to proton activation. In addition, concentration response curves for proton activation in the absence or presence of group A antagonists indicated a non-

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competitive inhibition of proton activation of rat TRPV1 i.e., curves shifted to the right in a non-parallel fashion with decreasing maximum (Supplemental figure 1).

Capsazepine and SB-366791 compete with and prevent BCTC antagonism, but not ruthenium red antagonism of rat TRPV1 activation by protons. Since capsazepine and SB-366791 are competitive antagonists of capsaicin activation, and were found to be ineffective at blocking proton (pH 5) activation of rat TRPV1, we investigated the possibility that capsazepine and SB-366791 fail to block proton activation because their affinity to rat TRPV1 is altered by proton activation-induced conformational changes in the capsaicin-binding pocket. We hypothesized that if indeed capsazepine or SB-366791 bind at the same binding pocket as BCTC, and proton activation does not alter the binding pocket, then they should compete with and prevent BCTC antagonism of rat TRPV1 activation by protons. We chose two concentrations of BCTC, one that blocks approximately 50% of pH 5-induced $^{45}Ca^{2+}$ uptake (0.5 nM), and a second one that blocks approximately 90% of pH 5-induced $^{45}Ca^{2+}$ uptake (2.5 nM). These BCTC concentrations were combined with different concentrations of either capsazepine (200 nM to 40 μM) or SB-366791 (200 nM to 40 μM) and the concentration-response relationship was determined in capsaicin- and pH 5-induced $^{45}Ca^{2+}$ uptake assays (Fig. 2).

As expected for additive antagonism, both capsazepine and SB-366791 blocked the remaining capsaicin-induced $^{45}Ca^{2+}$ uptake in the presence of BCTC (0.5 nM) in a concentration-dependent manner (Fig. 2A,B). Remarkably, when activating with protons, both capsazepine and SB-366791 prevented BCTC antagonism of rat TRPV1, as seen by a concentration-dependent increase of pH 5-induced $^{45}Ca^{2+}$ uptake in the presence of BCTC (Fig. 2C,D). These findings indicate that both capsazepine and SB-366791 compete with BCTC for binding to rat TRPV1 under low pH conditions, but do not block proton activation, as well as support the hypothesis that proton activation does not alter the capsaicin-binding pocket of rat TRPV1.

Both capsazepine and SB-366791 showed additive antagonism with ruthenium red, a pore blocker, at blocking rat TRPV1 activation by capsaicin, but they did not prevent

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ruthenium red antagonism of proton activation (Fig. 2E,F). As expected, this indicates that ruthenium red and capsazepine/SB-366791 do not compete at the same binding pocket.

Capsazepine and SB-366791 prevention of BCTC antagonism is competitive.
Capsazepine and SB-366791 were able to prevent BCTC antagonism of rat TRPV1 activation by protons, suggesting that they bind at the same site as BCTC under low pH conditions (pH 5). To clarify whether the mechanism of capsazepine and SB-366791 prevention of BCTC antagonism is competitive or non-competitive, we tested the concentration-response curve of BCTC antagonism of rat TRPV1 activation by protons in the absence or presence of different concentrations of either capsazepine or SB-366791 (Fig. 3A-D). As expected for compounds competing at the same binding pocket, capsazepine and SB-366791 caused parallel rightward shifts in the BCTC concentration-response (inhibition) curve, i.e. more BCTC was required to show the same level of inhibition in the presence of capsazepine or SB-366791. IC_{50} values for BCTC alone, or in the presence of 1.1, 3.3, and 10 μM of capsazepine were 0.36, 1.1, 2.9, and 13 nM respectively (Fig. 3A). Schild analysis determined a pA_2 value of 6.2 with a slope of 1.26 for capsazepine at the BCTC binding site (Fig. 3B). IC_{50} values for BCTC alone, or in the presence of 2, 6, and 18 μM of SB-366791 were 0.57, 2.9, 5.9, and 22.9 nM, respectively (Fig. 3C). Schild analysis gave a pA_2 value of 6.26 with a slope of 1.02 for SB-366791 at the BCTC binding site (Fig. 3D). Both capsazepine and SB-366791 showed no effect on the concentration-response curve for inhibition by ruthenium red (Fig. 3E and data not shown). An unrelated compound, A-317491 (a selective $P2X_{2/3}$ antagonist, Jarvis et al., 2002) showed no effect on either BCTC or ruthenium red antagonism of TRPV1 activation by protons (Fig. 3F and data not shown).

Antagonists from different chemical scaffolds compete at the same binding pocket.
Capsazepine, SB-366791 and BCTC belong to different chemical scaffolds yet compete for binding at the capsaicin-binding pocket of rat TRPV1 in a pH-independent manner, suggesting that proton activation does not alter the binding pocket (Fig. 1-3). To extend this observation to other chemical scaffolds, we have conducted antagonist competition

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experiments between each of the group A antagonists against each of the group B antagonists. First, we tested the ability of AMG0610 to compete with and prevent AMG6880, AMG7472, and BCTC antagonism of rat TRPV1 activation by protons. As expected for compounds competing at the same binding pocket, AMG0610 caused parallel rightward shifts in the inhibition curves of each of the group A antagonist (Figure 4, Table 3). Similarly, all group B antagonists competed with and caused parallel rightward shifts in each of the group A antagonist concentration-response inhibition curves of rat TRPV1 activation by protons, indicating that all of these antagonists compete with each other at the same binding pocket at pH 5 (Figure 4, and data not shown). The pA_2 values determined by Schild analysis for each of the group B antagonists at the each of the group A antagonist binding site are shown in Table 3.

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Discussion

By utilizing agonist-induced $^{45}Ca^{2+}$ uptake and CHO cells stably expressing rat TRPV1, we have characterized AMG0610 and AMG7472 as novel TRPV1 antagonists, confirmed the pharmacology of AMG6880, BCTC, and capsazepine and shown that all antagonists are competitive against capsaicin activation. Group A antagonists (AMG6880, AMG7472, and BCTC) blocked both capsaicin and proton activation, whereas group B antagonists (AMG0610, capsazepine, and SB-366791) blocked capsaicin, but not proton, activation of rat TRPV1. TRPV1 antagonists that block all modes of activation of rat (AMG9810, A-425619, BCTC) or guinea pig (capsazepine) TRPV1 showed efficacy in rat and guinea pig models of inflammatory pain, respectively (Gavva et al., 2005; Honore et al., 2005; Pomonis et al., 2003; Walker et al., 2003). Because antagonism of all modes of TRPV1 activation appears to correlate with anti-hyperalgesic effects *in vivo*, antagonists like AMG6880, AMG7472 and BCTC may help define the role of TRPV1 in pain and other disease states.

It should be noted that SB-366791 was reported to be an antagonist of both capsaicin and proton activation of rat and human TRPV1 in electrophysiological studies using transfected HEK293 cells (Gunthorpe et al., 2004). However, in $^{45}Ca^{2+}$ uptake assays using CHO cells we have previously shown that SB-366791 is an antagonist of capsaicin activation of both rat and human TRPV1, but is ineffective (IC_{50} >40 μM) against proton (pH 5) activation of both rat and human TRPV1 (Gavva et al., 2005). The key differences between these two studies are: i) non-transfected HEK293 cells show proton-activated currents, whereas CHO cells do not show any proton-induced $^{45}Ca^{2+}$ uptake, ii) electrophysiological assays are conducted in the absence of extracellular calcium that eliminates calcium-dependent desensitization of the TRPV1 channels, where as $^{45}Ca^{2+}$ uptake assays are conducted in the presence of extracellular calcium at a final concentration of 1mM and represents a more physiological environment compared to electrophysiological studies, and iii) potentiation of rat TRPV1 activation by protons observed in CHO cells may not be observed in electrophysiological assays if it occurs by delaying calcium dependent desensitization. Further studies should clarify the role of cell

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background and differences in antagonist pharmacology in electrophysiological versus $^{45}Ca^{2+}$ uptake assays. In addition, careful examination of the cDNA sequences used by different groups for the presence of single nucleotide polymorphisms in TRPV1 and their role in agonist and antagonist sensitivity should help reveal underlying mechanism(s) for the observed conflicting results using TRPV1 antagonists like capsazepine (McIntyre et al., 2001; Seabrook et al., 2002), iodo-resiniferatoxin (Seabrook et al., 2002; Shimizu et al., 2005) and SB-366791 (Gunthorpe et al., 2004; Gavva et al., 2005).

Using chimeric domain swap analysis between capsaicin-sensitive TRPV1 and capsaicin-insensitive TRPV1 (Gavva et al., 2004; Jordt and Julius 2002), it was shown that vanilloid sensitivity and [^{3}H]-resiniferatoxin binding are transferable with the TM3/4 region, suggesting that the TM3/4 region constitutes the vanilloid binding pocket. Further, mutagenesis studies within the TM3/4 region identified several key molecular determinants for both agonist and antagonist actions (Chou et al., 2004; Gavva et al., 2004; Jordt and Julius 2002; Phillips et al., 2004), and led to the proposed models of the vanilloid-binding pocket (Chou et al., 2004; Gavva et al., 2004; Jordt and Julius, 2002). Here, we show that all of the group A and B antagonists are competitive against capsaicin activation, require the same critical molecular determinants, and appear to interact at the same binding pocket. It is, however, not clear if the differential pharmacologies of AMG0610, capsazepine, and SB-366791 are due to their altered affinity caused by proton activation-induced conformational changes in the capsaicin-binding pocket.

We hypothesized that if group B antagonists bind at the same site as group A antagonists on rat TRPV1 and proton activation does not alter the capsaicin-binding pocket, then they should be able to compete with and prevent group A antagonism of rat TRPV1 activation by protons. We have shown that group B antagonists act additively with group A antagonists to block capsaicin activation. Remarkably, each of the group B antagonists prevented all of the group A antagonists, from blocking proton activation of rat TRPV1, indicating specific competition between group A and B antagonists at pH 5. This proves unequivocally that group B antagonists bind rat TRPV1 at pH 5 with similar affinity as they do at pH 7.2 during capsaicin activation (compare pA_2 values in Table 1 and 3), but

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that they are ineffective against blocking proton activation. It also confirms that all antagonists (group A and B) interact at the same or an overlapping binding pocket. Finally, it suggests that proton activation neither alters the capsaicin-binding pocket nor affects antagonist affinity at the capsaicin-binding pocket. The pA_2 values of each of the three group B antagonists at each of the group A antagonist binding sites are similar (Table 3), further showing that indeed all these antagonists bind to the same pocket and their affinities are not altered by proton activation of TRPV1. These results also suggest that acidification does not affect group B antagonists and their ability to bind rat TRPV1. Furthermore, calculated pKa values suggest acidification may not affect group B antagonists (data not shown). None of the group B antagonists competed with or affected ruthenium red inhibition of TRPV1, indicating that there is no overlap in the binding pocket for competitive antagonists (group A and B) and the pore blocker, ruthenium red.

We propose that both agonists and antagonists share the same binding pocket (Figure 5) and that proton activation does not alter the conformation of the binding pocket based on i) the competitive antagonism of capsaicin activation by all of these antagonists (Gunthorpe et al., 2004; Valenzano et al., 2003 and this study), ii) the requirement of the same critical determinants for actions of all antagonists (Gavva et al 2004 and this study), iii) the ability of AMG0610, capsazepine and SB-366791 to compete with each of the group A antagonists and iv) the similarity of pA_2 values of group B antagonists at the capsaicin-binding pocket at pH 7.2 (capsaicin activation) and pH 5 (proton activation). Since the critical molecular determinants for capsaicin or vanilloid interaction (Gavva et al., 2004, Jordt and Julius, 2002) and proton activation (Jordt et al., 2000) have been reported to be different, agonism of TRPV1 by protons should be considered allosteric to the capsaicin site. The ability to block proton activation of TRPV1 by competitive antagonists that interact at the capsaicin-binding pocket occurs perhaps by locking the channel conformation in a non-conducting state. We propose that these antagonists should be defined as allosteric inhibitors for proton activation. For example, group A antagonists are competitive antagonists at the capsaicin-binding pocket and allosteric inhibitors for proton activation, whereas group B antagonists are just competitive antagonists at the capsaicin binding pocket of TRPV1 (Figure 5). No allosteric inhibition

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of rat TRPV1 activation by protons was seen with group B antagonists, even though they bind to rat TRPV1 in the same binding pocket as group A antagonists (Figure 5). Based on these observations, we propose that group A antagonists stabilize or lock the channel conformation in a closed or non-conducting state when they interact at the capsaicin binding pocket, whereas group B antagonists only act as true competitive antagonists at the capsaicin binding pocket but do not lock or stabilize the non-conducting state of the channel.

Notably, all TRPV1 antagonists reported to date appear to bind at the capsaicin site (competitive antagonists of capsaicin activation), and based on their ability to lock or stabilize the channel conformation, some are able to block all modes of activation. Other than the non-selective pore blocker ruthenium red, compounds that block all modes of TRPV1 activation via interaction at a site outside of the capsaicin-binding pocket have not been identified yet. The trialkylglycine-based compounds DD161515 and DD191515 have been reported as non-competitive antagonists of capsaicin activation that may act through a different site than capsaicin (Garcia-Martinez et al., 2002). However, it is not known whether these molecules block all modes of TRPV1 activation. Ruthenium red appears to block all modes of TRPV1 activation by interacting with the residues in the channel pore (Garcia-Martinez et al., 2000).

Interestingly, capsazepine, although classified as a group B antagonist at rat TRPV1, acts as a group A antagonist at human and guinea pig TRPV1 as well as the capsaicin-sensitive rabbit TRPV1 mutant, I550T, i.e., capsazepine blocks proton activation of these channels (Gavva et al., 2004; McIntyre et al 2001; Savidge et al., 2002). Although capsazepine is a weak antagonist of capsaicin activation of rat TRPV1, it appears that it is not able to lock or hold the channel conformation in the closed state since it is ineffective against proton activation of rat TRPV1. However, depending on the pharmacokinetic properties, molecules like capsazepine may act as anti-hyperalgesics in humans since it is a potent antagonist at all modes of human TRPV1 activation. To be able to predict efficacy in humans, it is important to identify a suitable animal species for *in vivo*

pharmacology studies based on the comparable antagonism profile at TRPV1 from such species and humans.

The ability to block proton activation of TRPV1 by capsazepine requires Leu^{547}, because replacement of Leu^{547} with Met^{547} in the capsaicin-sensitive rabbit TRPV1 mutant (rabbit TRPV1-I550T; Gavva et al., 2004) or in human TRPV1 (Phillips et al., 2004) resulted in capsazepine being ineffective against proton activation. Conversely, mutations in the TM3/4 region of rat TRPV1 to the corresponding human TRPV1 residue (I514M, V518L, and M547L) enabled capsazepine inhibition of proton activation at a level similar to that observed in human TRPV1 (Phillips et al., 2004). AMG6880 and AMG9810 block proton activation of TRPV1 with either Leu^{547} (human TRPV1 and rabbit TRPV1-I550T) or Met^{547} (rat TRPV1), whereas AMG0610 and SB-366791 are ineffective against blocking proton activation of TRPV1 with either Leu^{547} (human TRPV1 and rabbit TRPV1-I550T) or Met^{547} (rat TRPV1; Table 2, Gavva et al., 2005, and data not shown). These studies indicate that a single residue within the capsaicin-binding pocket can differentially affect the ability of TRPV1 antagonists to lock or stabilize the channel conformation in the closed state. The mechanisms of action for blocking all modes of TRPV1 activation appears to be more complex due to the fact that molecules interacting at the capsaicin-binding pocket act either as agonists, potentiators of proton activation or antagonists for some or all modes of activation. For example, some group B antagonists such as AMG-0610, SB-366791 (Fig. 1C), and KJM429 (Wang et al., 2002) act as potentiators of proton activation of rat TRPV1, probably by increasing the open channel conformation or by delaying desensitization of the TRPV1 channel. Still to be determined are the precise interactions of specific residues within the capsaicin-binding pocket with different antagonists and their role in locking or stabilizing the channel conformation in the closed or non-conducting state and/or interfering with the inactivation and/or desensitization of TRPV1.

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Figure Legends

Figure 1. Structures of antagonists used in the study are shown in (**A**). Comparison of antagonists for inhibition of (**B**) capsaicin (0.5 μM) and (**C**) proton (pH 5) induced activation of rat TRPV1. CHO cells stably expressing rat TRPV1 were used in agonist induced $^{45}Ca^{2+}$ uptake assay as described under *Materials and Methods*. Cells were incubated for 2 minutes with increasing concentrations of antagonists as indicated, followed by the addition of agonists for additional 2 minutes. Each point in the graph are average $\pm$ SD of an experiment conducted in triplicate. Please note potentiation of pH 5-induced $^{45}Ca^{2+}$ uptake above 100% by SB-366791 at high concentrations (>10 μM) in **C.** IC_{50} values for capsaicin and pH 5 activation are shown in Table 2. (**D**) Concentration-response curves for capsaicin-induced $^{45}Ca^{2+}$ uptake into CHO cells expressing rat TRPV1 in the absence or presence of 1.11, 3.33 or 10 μM capsazepine. (**F**) Schild analysis of the antagonism produced by 1.11-10 μM capsazepine.

Figure 2. Concentration dependent inhibition of capsaicin activation by mixtures of capsazepine and BCTC (**A**) or SB-366791 and BCTC (**B**) or capsazepine and ruthenium red (RR; **E**). Note the remaining capsaicin-induced $^{45}Ca^{2+}$ uptake in the presence of BCTC (0.5 nM) or 150 nM RR inhibition was concentration dependently inhibited by capsazepine and SB-366791 indicating additive antagonism. Capsazepine (**C**) and SB-366791 (**D**) compete with BCTC and prevent BCTC inhibition of rat TRPV1 activation by protons. Note that BCTC inhibition of proton-induced $^{45}Ca^{2+}$ uptake is concentration-dependently prevented by both capsazepine and SB-366791 (**C**, **D**), however, capsazepine did not affect RR inhibition of proton-induced $^{45}Ca^{2+}$ uptake (**F**). IC_{50} values for capsazepine and SB-366791 blockade of 2.5 nM BCTC inhibition of rat TRPV1 activation by protons are, 0.55 $\pm$ 0.07 μM, and 1.15 $\pm$ 0.09 μM respectively. At higher concentrations (>0.3 μM), SB-366791 potentiation is represented by an increase of $^{45}Ca^{2+}$ uptake above 100% (**D**).

Figure 3. (**A).** Concentration-response curves for BCTC inhibition of proton-induced $^{45}Ca^{2+}$ uptake into CHO cells expressing rat TRPV1 in the absence or presence of 1, 3 or

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10 μM capsazepine. **(B).** Schild analysis of competitive interaction between BCTC and 1.11-10 μM capsazepine. (**C)**. Concentration response curves for BCTC inhibition of proton-induced $^{45}Ca^{2+}$ uptake into CHO cells expressing rat TRPV1 in the absence or presence of 3, 10 or 30 μM SB-366791 **(D).** Schild analysis of competitive interaction between BCTC and 2-18 μM SB-366791.

Figure 4. (**A**). Concentration response curves for AMG6880 inhibition of proton-induced $^{45}Ca^{2+}$ uptake into CHO cells expressing rat TRPV1 in the absence or presence of 0.3,1, 3.3, or 10 μM AMG0610. (**B)**. Schild analysis of competitive interaction between AMG6880 and 0.3-10 μM AMG0610. (**C)**. Concentration response curves for AMG7472 inhibition of proton- induced $^{45}Ca^{2+}$ uptake into CHO cells expressing rat TRPV1 in the absence or presence of 0.1 – 3.3 μM AMG0610. (**D)**. Schild analysis of competitive interaction between AMG7472 and 0.13.3 μM AMG0610. (**E)**. Concentration response curves for BCTC inhibition of proton- induced $^{45}Ca^{2+}$ uptake into CHO cells expressing rat TRPV1 in the absence or presence of 0.3 – 10 μM AMG0610. (**F)**. Schild analysis of competitive interaction between BCTC and 0.3-10 μM AMG0610.

Figure 5. Models of agonist and antagonist interaction with capsaicin-binding pocket of rat TRPV1. Left panel: Capsaicin binds the intra-membrane vanilloid-binding pocket constituted by the TM3/4 region. Antagonists such as AMG0610, AMG6880, AMG7472, BCTC, capsazepine and SB-366791 compete with capsaicin for the same binding pocket. Middle panel: Critical residues for proton activation are located at the pre-pore loop. Antagonists like AMG6880, AMG7472, and BCTC (group A) bind at the capsaicin-binding pocket and stabilizes or locks the channel conformation in the closed or non-conducting state even when protons are present at pH 5 (it is not known if group A antagonist binding prevents protonation of TRPV1). Right panel: AMG0610, capsazepine and SB-366791 (group B) bind the same pocket as group A antagonists but do not stabilize or lock channel confirmation in the closed or non conducting state. Proton activation (pH 5-induced conformational changes) occurs while the group B antagonists are bound to the channel and $^{45}Ca^{2+}$ passes through. Capsaicin and protons

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are shown in red and green squares, respectively. All antagonists (circles) are shown in green in the left panel, group A antagonists are shown in yellow (middle panel) and group B antagonists are shown in blue (right panel).

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Table 1: The pA_2 values of TRPV1 antagonists at the capsaicin-binding site determined using CHO cells stably expressing rat TRPV1 and capsaicin-induced $^{45}Ca^{2+}$ uptake assay.

Group A antagonists	**pA2**	**Slope**
AMG-6880	8.25 ± 0.32	-0.995 + 0.074
AMG-7472	9.14 ± 0.25	1.084 ± 0.184
BCTC	9.21 ± 0.13	1.256 ± 0.095
Group B antagonists	**pA2**	**Slope**
AMG-0610	7.59 ± 0.27	1.214 ± 0.202
Capsazepine	6.07 ± 0.14	1.615 ± 0.026
SB-366791	6.02 ± 0.08	1.119 ± 0.135

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Table 2: Molecular determinants for antagonist action are located in the TM3/4 region of TRPV1. IC_{50} values for antagonists at the pH 5 and capsaicin activation induced $^{45}Ca^{2+}$ uptake in to CHO cells stably expressing TRPV1 are shown in nanomolar ± SD.

	pH 5 activation			**Capsaicin activation**	
Antagonist	Rat	Rabbit	Rabbit-I550T	Rat	Rabbit-I550T
Group A					
AMG6880	24 ± 3	>4000	167 ± 73	11 ± 6	3 ± 0.2
AMG7472	2 ± 0.7	>4000	7 ± 2	1 ± 0.6	3 ± 1
BCTC	0.6 ± 0.1	>400	1.3 ± 0.2	0.4 ± 0.1	2 ± 1
Group B					
AMG0610	>4000	>4000	>4000	23 ± 3	24 ± 5
Capsazepine	>4000	>4000	421 ± 208	659 ± 155	477 ± 225
SB-366791	>4000	>4000	>4000	937 ± 207	720 ± 203
$P2X_{2/3}$					
A-317491	>4000	>4000	>4000	>4000	>4000

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Table 3: The pA_2 values of group B antagonists (AMG0610, capsazepine, and SB-366791) at the binding sites of group A antagonists (AMG6880, AMG7472 and BCTC) determined in pH 5-induced $^{45}Ca^{2+}$ uptake into CHO cells stably expressing rat TRPV1. Slope values are shown in parenthesis.

	AMG0610	Capsazepine	SB-366791
AMG7472	7.30 ± 0.10 (0.927 ± 0.017)	6.12 ± 0.07 (0.976 ± 0.132)	6.58 ± 0.18 (0.959 ± 0.070)
BCTC	6.78 ± 0.18 (1.027 ± 0.041)	6.37 ± 0.10 (1.087 ± 0.087)	6.33 ± 0.27 (1.023 ± 0.168)
AMG6880	6.71 ± 0.04 (1.116 ± 0.090)	6.44 ± 0.22 (0.979 + 0.031)	6.32 ± 0.13 (1.050 ± 0.258)

Figure 1

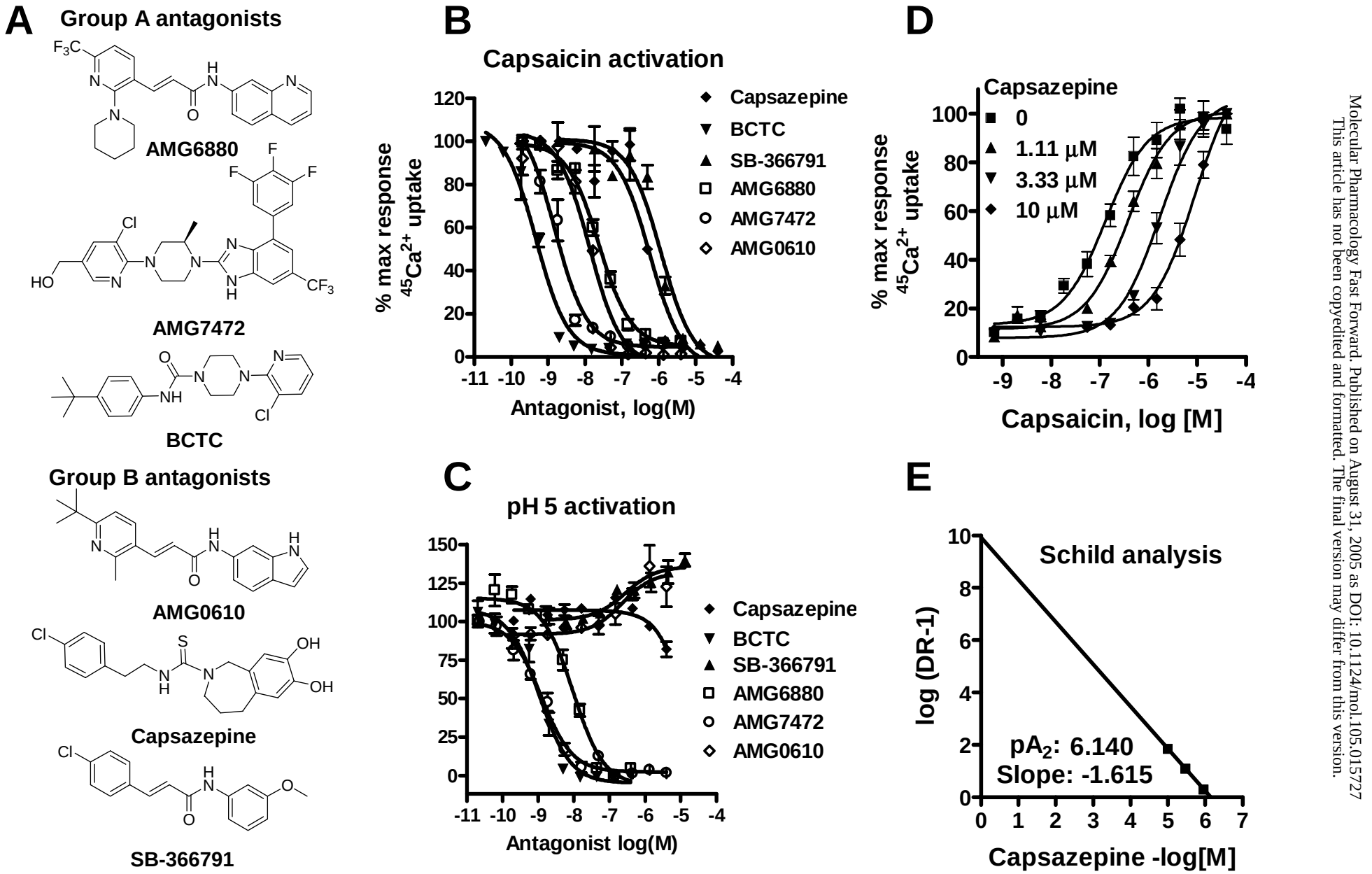

Figure 2

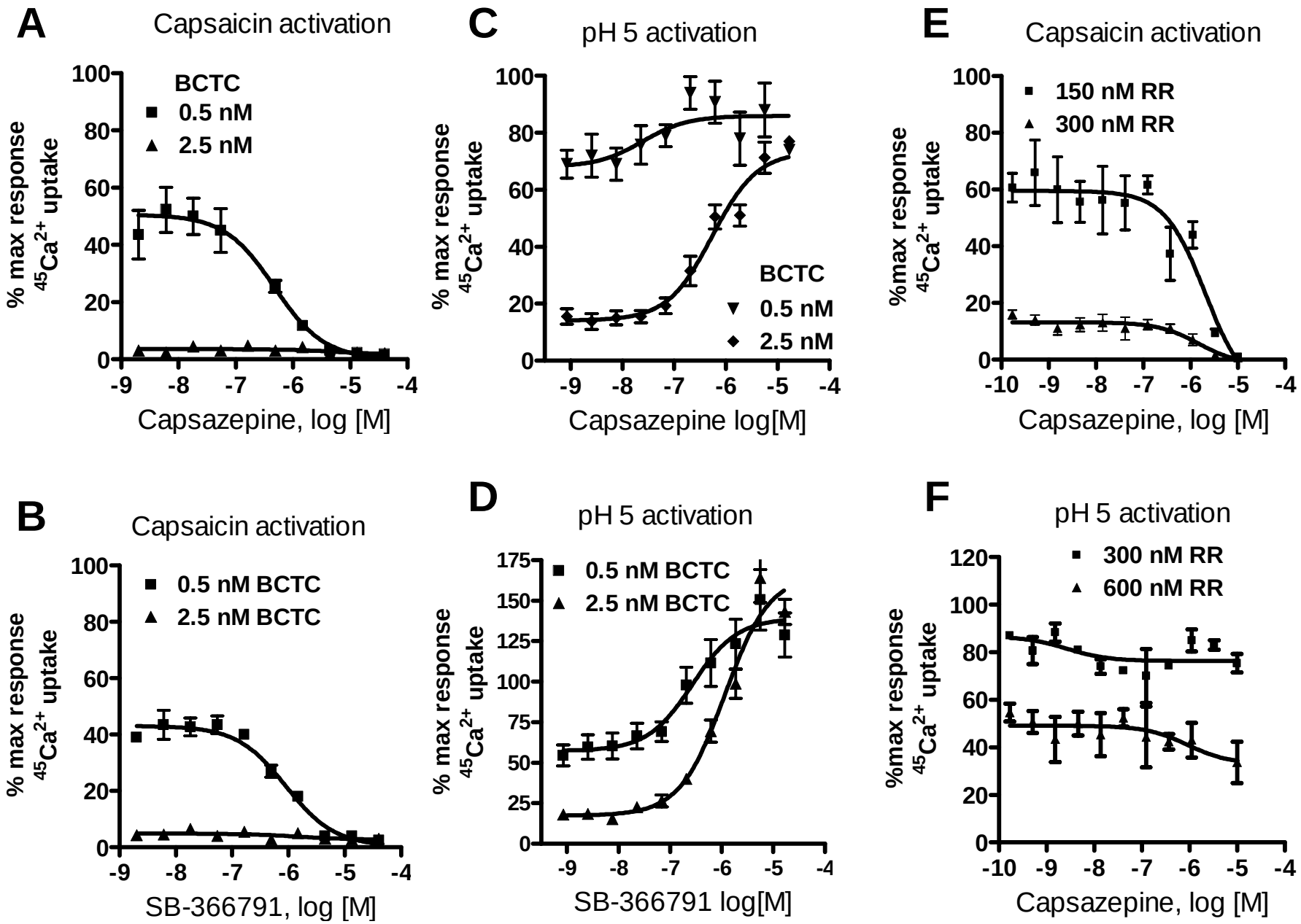

Figure 3

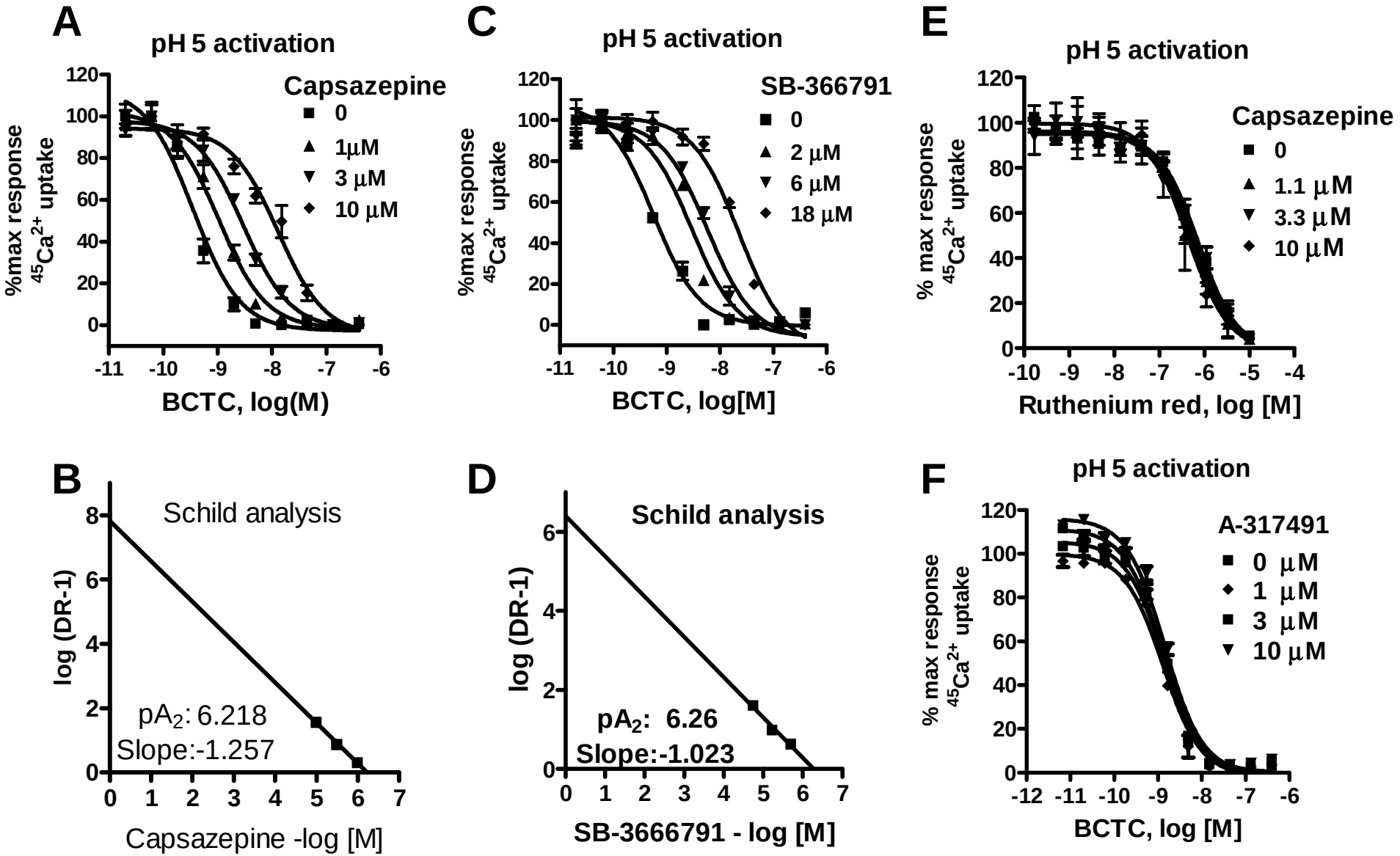

Figure 4

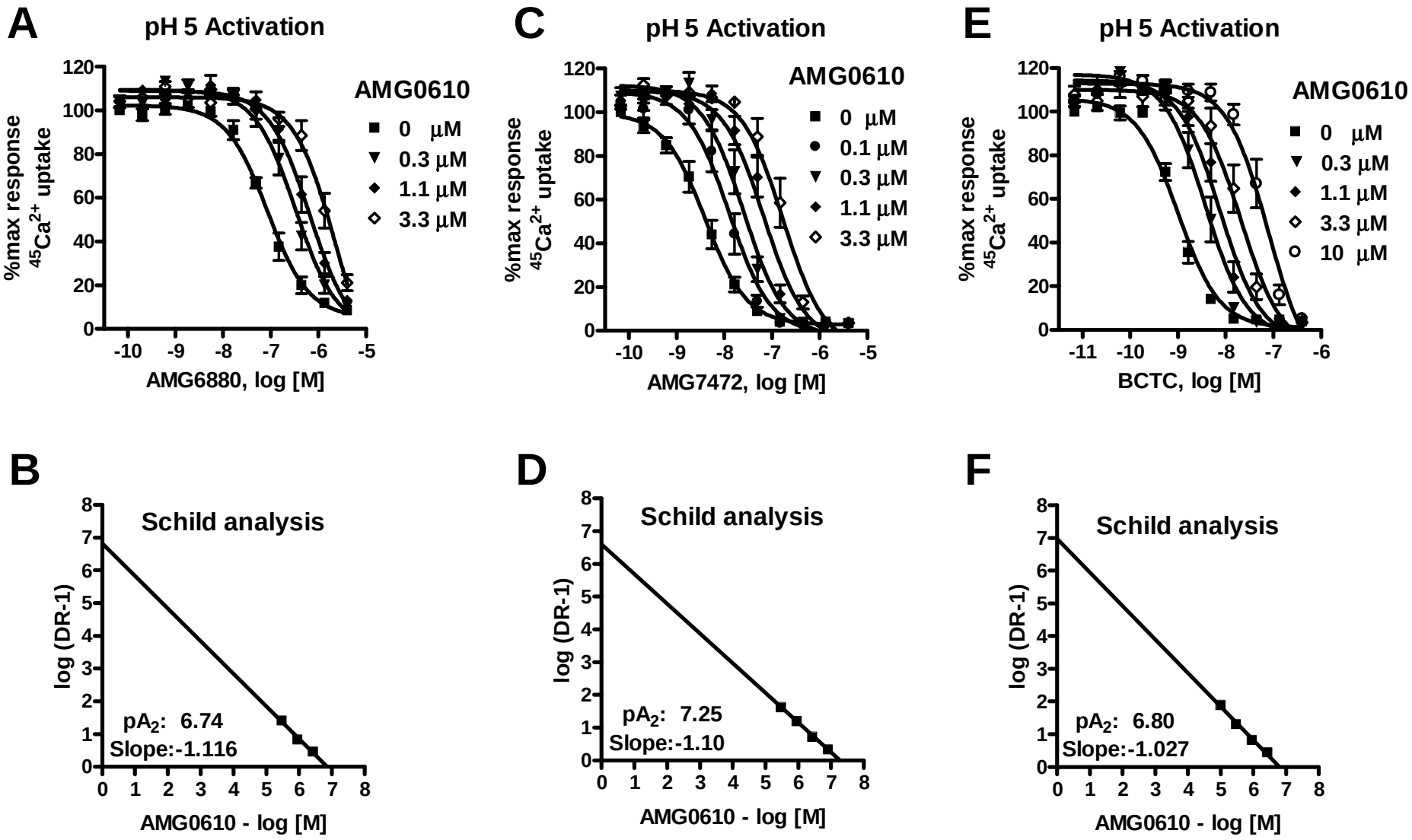

Figure 5