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A polyclonal antibody to the pre-pore loop of TRPV1 blocks channel activation

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The abbreviations used are: VR1 or TRPV1, transient receptor potential vanilloid type 1; CHO, chinese hamster ovary; TM3/4, transmembrane 3-4 region; A-425619, 1-isoquinolin-5-yl-3-(4-trifluoromethyl-benzyl)-urea; AMG9810, (E)-3-(4-t-butylphenyl)-N-(2,3-dihydrobenzo[b][1,4] dioxin-6-yl)acrylamide; AMG6880, (2*E*)-3-[2-piperidin-1-yl-6-(trifluoromethyl)pyridin-3-yl]-*N*-quinolin-7-ylacrylamide; AMG7472, 5-chloro-6-{(3*R*)-3-methyl-4-[6-(trifluoromethyl)-4-(3,4,5-trifluorophenyl)-1*H*-benzimidazol-2-yl]piperazin-1-yl}pyridin-3-yl)methanol; AMG0347, (E)-N-(7-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl)-3-(2-(piperidin-1-yl)-6-(trifluoromethyl)pyridin-3-yl)acrylamide; BCTC, *N*-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2*H*)-carboxamide; JNJ-17203212, 4-(3-(trifluoromethyl)pyridin-2-yl)-N-(5-(trifluoromethyl)pyridin-2-yl)piperazine-1-carboxamide

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Abstract

The Vanilloid receptor 1 (TRPV1) can be activated by multiple chemical and physical stimuli such as capsaicin, anandamide, protons and heat. Capsaicin interacts with the binding pocket constituted by transmembrane regions 3 and 4, whereas protons act through residues in the pre-pore loop of TRPV1. Here we report on characterization of polyclonal and monoclonal antibodies to the pre-pore loop of TRPV1. A rabbit anti-rat TRPV1 polyclonal antibody (Ab-156H) acted as a full antagonist of proton activation (IC_{50} values for pH 5 and 5.5 were 364.68 $\pm$ 29.78 nM and 28.31 $\pm$ 6.30 nM, respectively) and as a partial antagonist of capsaicin, heat, and pH 6 potentiated chemical ligand (anandamide and capsaicin) activation (50-79% inhibition). Ab-156H antagonism of TRPV1 is not affected by the conformation of the capsaicin-binding pocket, because it is equally potent at wild type (capsaicin-sensitive) rat TRPV1 and its T550I mutant (capsaicin-insensitive). With the goal of generating monoclonal antagonist antibodies to the pre-pore region of human TRPV1, we used a recently developed rabbit immunization protocol. Whereas rabbit polyclonal antiserum blocked human TRPV1 activation, rabbit monoclonal antibodies (identified on the basis of selective binding to CHO cells expressing human TRPV1) did not block activation by either capsaicin or protons. Thus, rabbit polyclonal antibodies against rat and human TRPV1 pre-pore region appear to partially lock or stabilize the channel in the closed state, whereas rabbit anti-human TRPV1 monoclonal antibodies bind to the pre-pore region but do not lock or stabilize the channel conformation.

Introduction

Transient receptor potential vanilloid type 1 (*a.k.a.* VR1 or TRPV1), a non-selective cation channel, is activated by chemical ligands (capsaicin, anandamide, and protons [pH $\leq$ 5.7]) and heat (>42 ºC), acting as an integrator of multiple noxious stimuli (Caterina, et al., 1997; Holzer, 2004; Szallasi and Blumberg, 1999; Szolcsanyi, 2004; Tominaga et al., 1998). 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 reduce the threshold of activation of nociceptors that express TRPV1, resulting in 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).

Several competitive antagonists of TRPV1 that interact with the capsaicin/vanilloid-binding pocket have been reported to prevent activation of the channel by different stimuli (reviewed in Szallasi and Appendino, 2004). These antagonists include those that inhibit hyperalgesia in models of inflammatory pain (AMG9810, Gavva et al., 2005a; A-425619, Honore et al., 2005; and BCTC, Valenzano et al., 2003), skin incision-induced pain (A-425619, Honore et al., 2005; AMG0347, Gavva et al, 2005b), and bone cancer pain (JNJ-17203212, Ghilardi et al., 2005) supporting a role for TRPV1 in clinical pain states.

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 capsaicin/vanilloid sensitivity and [^{3}H]-resiniferatoxin binding were 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 such as Tyr^{511}, Ser^{512},Met/Leu^{547}, Thr^{550} that led to the proposed models of agonist and antagonist interactions with the vanilloid-binding pocket (Chou et al., 2004; Gavva et al., 2004; Jordt and Julius 2002; Lee et al., 2005). Molecular determinants for proton activation have been reported to be present in the pre-pore loop, such as Glu^{600} and Glu^{648} (Jordt et al., 2000) and are different from those that constitute the vanilloid-binding pocket. Competitive antagonists of TRPV1, such

as AMG6880, AMG7472 and BCTC that interact at the vanilloid-binding pocket appear to lock the channel conformation in the closed state to block all modes of activation (Gavva et al., 2005c). Ruthenium red, a non-selective pore blocker, acts as an antagonist of all modes of TRPV1 activation via interaction with residues in the channel pore, such as Asp^{646} (Garcia-Martinez et al., 2000).

Antibodies are generally peripherally-restricted, should be devoid of potential central side-effects, and with their long plasma half-life might be better therapeutic agents for chronic disease conditions such as pain. Hence, we used the pre-pore loop peptide of TRPV1 as an antigen to generate antagonist antibodies. We used agonist-induced $^{45}Ca^{2+}$ uptake assays and CHO cells expressing TRPV1 to show that rabbit anti-TRPV1 polyclonal, but unfortunately not monoclonal, antibodies act as antagonists.

Methods

Cloning and stable transfections. Cloning and stable cell generation for rat TRPV1 was described in Gavva et al., (2004). Stable expression of human TRPV1 was achieved by transfection of AM1-D CHO cells (US patent #6,210,924) with a pDSRα21 expression construct. Transfection was performed using LF2000 (Invitrogen) according to the manufacturer's protocol. Briefly, 4 x 10^6AM1-D CHO cells were plated 24 hours prior to transfection, in 100 mm diameter plastic petri dishes (Falcon) in 10 ml of Dulbecco's Modified Eagles Medium (Invitrogen) supplemented with 5% fetal bovine serum, 1x penicillin-streptomycin and glutamine (Invitrogen), non-essential amino acids (Invitrorgen), sodium pyruvate, and HT (Invitrogen). Approximately 30 μg of each pDSRα21 – human TRPV1 plasmid DNA was linearized using the restriction enzyme PvuI (NEB) and diluted in 2 ml of OptiMEM (Invitrogen). The diluted DNAs were mixed with 75 μl of LF2000 diluted in 2 ml of OptiMEM, and the mixture was incubated for 20 minutes at room temperature. The DNA/LF2000 mixture was added to the cells and incubated overnight for transfection. The following day, fresh growth medium was added and cells were cultured for 48 hours, then plated in selection medium, containing DMEM supplemented with 10% dialyzed fetal bovine serum, 1x penicillin-streptomycin and glutamine, non-essential amino acids (Invitrogen), and sodium pyruvate, in 1:10 dilution. Approximately 2 weeks after transfection, surviving cells were plated one cell per well into five 96-well plates, using limited dilution techniques. Chinese hamster ovary (CHO) cells stably expressing rat TRPV1 were maintained in DMEM supplemented with 10% dialyzed FBS, 800 μg/ml Genetecin, penicillin, streptomycin, L-glutamine, and non-essential amino acids. HEK293 cells transiently transfected with human TRPV1 cDNA using Fugene were used for some of the whole cell ELISA experiments.

Generation of rabbit polyclonal antibodies to pre-pore loop of rat TRPV1. Synthetic peptide corresponding to Glu^{600} to Pro^{623} (EDGKNNSLPMESTPHKCRGSACKP) was coupled to KLH and used to generate polyclonal antibodies following the standard procedures. A peptide affinity column was made by covalently coupling 1.76 mg of the synthetic antigen peptide to 2 mL of Pierce SulfoLink Coupling Gel. 10 mL of serum diluted with 20 mL of PBS was applied to the column by gravity flow. The column was washed with 20 mL of PBS, then the antibodies

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were eluted with 3 x 4.0 mL of Pierce ImmunoPure IgG Elution Buffer. Each fraction was neutralized with 160 µL of 1 M Tris, pH 9.2 buffer, concentrated in a 20 mL VivaSpin 10 kDa cut-off unit, and dialysed against PBS.

Generation of rabbit monoclonal antibodies to pre-pore loop of human TRPV1. Monoclonal antibodies to pre-pore loop of human TRPV1 (Thr^{598}LIEDGKND SLPSESTSH RWRGPACRP PDSSYNSLY STCys^{636}) were generated by Epitomics (Burlingame, CA) as a contract service, following the published protocols (Spieker-Polet et al., 1995). Conditioned media (45-48 mL) from hybridomas was mixed batchwise over night with 50 µL of Protein A-Sepharose FF from GE Healthcare. The gel was transferred to 0.5 mL SpinX filter tubes and the gel was washed (5 x 250 µL PBS) and eluted (3 x 75 µL Pierce IgG ImmunoPure Elution Buffer) by spinning in an Eppendorf 5415 centrifuge. Each fraction was neutralized with 3 µL of 1 M Tris, pH 9.2.

Whole cell ELISA. CHO cells expressing TRPV1 and parental CHO cells were grown to confluency in 96 well plates at 37^{o}C. ELISA was done at room temperature. Growth medium was aspirated and cells were incubated with blocking buffer (2% milk in PBS w/$Ca^{2+}$and Mg^{2+}) for 1 hr. Blocking buffer was removed and cells were incubated with purified antibodies (at 5 µg/ml in blocking buffer) or conditioned media (at a 1:1 ratio in blocking buffer) for 1 hr. Cells were washed 3 times with wash buffer (PBS supplemented with 0.05%Tween) and then incubated with a secondary biotinylated antibody in blocking buffer at a 1:2000 dilution (cat #BA-1000, Vector Laboratories) for 1 hr. After 3 more washes, a Eu^{3+}-Streptavidin based DELFIA detection system (Cat#1244, Perkin-Elmer, CA) was used to measure time resolved fluorescence on a 96 well plate reader (Victor, Perkin-Elmer, CA).

$^{45}Ca^{2+}$ 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 was followed as a function of cellular uptake of radioactive calcium ($^{45}Ca^{2+}$, ICN). All the antagonist $^{45}Ca^{2+}$ uptake assays had a final $^{45}Ca^{2+}$ concentration of 10 µCi/mL. Antagonist assay of capsaicin activation. Purified antibodies were pre-incubated with TRPV1 expressing CHO cells for 2 hrs at 37^{o}C prior to addition of $^{45}Ca^{2+}$ and capsaicin (final concentration, 0.5 µM) in F12 media and then

incubated for an additional 2 minutes at room temperature. Antagonist assay of proton activation. Purified antibodies were pre-incubated with TRPV1 expressing CHO cells for 2hrs at 37^{o}C 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 or 5.5) and then incubated for an additional 2 minutes. Antagonist assay of heat activation. Purified antibodies were pre-incubated with TRPV1 expressing CHO cells for 2hrs at 37^{o}C prior to addition of $^{45}Ca^{2+}$ in F12 media supplemented with 30 mM HEPES, and then incubated for an additional 5 minutes on a heat block to reach a final temperature of 45^{o}C. Assay termination and data analysis**.** Immediately after the 2min incubation with agonist, 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 was measured using a MicroBeta Jet (Perkin-Elmer Inc.). Data were analyzed using Graphpad Prism 4.01 (GraphPad Software Inc, San Diego, CA).

Reagents. All cell culture reagents were purchased from Invitrogen (Carlsbad, CA).

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Results

A rabbit polyclonal antibody to rat TRPV1 pre-pore peptide acts as an antagonist

To test whether antibodies specific for peptides around the pore region of rat TRPV1 can block channel activation, we first examined an affinity-purified polyclonal antibody (Ab-156H) generated against a 23 amino acid peptide, corresponding to residues Glu^{600} to Pro^{623} (Fig.1A). This sequence of amino acids (EDGKNNSLPMESTPHKCRGSACKP), located between transmembrane region 5 and the pore-forming region, includes one residue, Glu^{600}, that was reported to mediate proton activation of TRPV1. A time resolved fluorescencebased ELISA demonstrated the binding of affinity-purified polyclonal antibody Ab-156H to rat TRPV1 expressed transiently in HEK293 and stably in CHO cells (Fig.1B and data not shown). Antigen peptide (Glu^{600} to Pro^{623}), but not an unrelated peptide, blocked the binding of Ab-156H to TRPV1 expressing cells, confirming the specificity of antibody (Fig.1B and data not shown). Also, Ab-156H did not bind to either of the parental cell lines. Although the pre-pore region of TRPV1 (Glu^{600} to Pro^{623}) is somewhat conserved across different species (Fig.1A), Ab-156H did not recognize mouse, rabbit, or human TRPV1 using immunostaining and/or ELISA techniques (data not shown).

Since the Glu^{600} to Pro^{623} region includes residues mediating proton activation of TRPV1, we first evaluated the ability of Ab-156H to inhibit proton-induced $^{45}Ca^{2+}$ uptake in CHO cells expressing rat TRPV1. Ab-156H inhibited both pH 5 and pH 5.5-induced $^{45}Ca^{2+}$ uptake in a concentration-dependent manner with IC_{50} values (mean $\pm$ SD, n=4) of 364.7 $\pm$ 29.8 nM and 28.3 $\pm$ 6.3 nM, respectively (Fig 2A). In addition, we tested the ability of the antigen peptide (Glu^{600} to Pro^{623}) to compete with and prevent Ab-156H antagonism of proton activation in a $^{45}Ca^{2+}$ uptake assay (Fig.2B). Preincubation of 20 μM antigen peptide with 2.7 μM Ab-156H prior to the pH 5 - induced $^{45}Ca^{2+}$ uptake completely eliminated Ab-156H antagonism of proton activation, further confirming the specificity of Ab-156H. It is possible that Ab-156H inhibition of proton activation occurs either by blocking protonation of the residues responsible for activation or by locking or stabilizing the channel in the closed state.

We hypothesized that if Ab-156H inhibition of TRPV1 activation by protons occur simply by occluding the residues from protonation, this antibody will not block capsaicin or anandamide activation that occurs through vanilloid-binding pocket located in the TM3/4 region. To test this hypothesis, we evaluated the effect of Ab-156H antibody on capsaicin- and anandamide-induced $^{45}Ca^{2+}$ uptake by CHO cells expressing rat TRPV1 (Fig 2C). Ab-156H inhibited both capsaicin- and anandamide-induced $^{45}Ca^{2+}$ uptake in a concentration-dependent manner with maximum inhibition of approximately 55% at the highest concentration tested (1 μM), suggesting that Ab-156H may partially lock the channel conformation in the closed state. In agreement with this, Ab-156H partially inhibited (approximately 55%) heat-induced $^{45}Ca^{2+}$ uptake in to CHO cells expressing rat TRPV1 (Fig.2D).

Since protons directly activate TRPV1 at pH $\leq$ 5.7 and potentiate capsaicin activation at pH 6.0 (Jordt et al., 2000; Ryu et al., 2003), we were curious to see if Ab-156H could block proton-potentiated capsaicin activation. We evaluated the effect of two concentrations of Ab-156H (10, 100 μg/ml) against pH 5.5, and capsaicin (0.1 μM) at pH 7.2 and at pH 6.0 (Fig.2E). Compared to activation at pH 7.2, capsaicin at pH 6.0 caused a 3-fold higher net $^{45}Ca^{2+}$ uptake into CHO cells expressing TRPV1, indicating potentiation by pH 6.0. Ab-156H blocks both non-potentiated and pH 6.0 potentiated capsaicin activation (Fig. 2E). It appears that Ab-156H blocks pH 6.0 potentiated capsaicin activation better than capsaicin activation at pH 7.2, suggesting that Ab-156H blocks not only the direct activation of TRPV1 by protons, but also potentiation of agonists by protons as well.

Ab-156H acts as a full antagonist of proton activation of both capsaicin-sensitive and –insensitive rat TRPV1 channels

We have previously shown that agonists and competitive antagonists require Thr^{550} for their actions at TRPV1. Mutation of Thr^{550} to Ile^{550} (T550I) renders rat TRPV1 insensitive to capsaicin and anandamide, as well as to antagonists such as AMG9810 and BCTC. (Gavva et al., 2004; Gavva et al., 2005a). The ability of protons to activate the TRPV1 channel was not altered by the T550I mutation. To investigate if the T550I mutation affects the ability of Ab-156H to block proton activation, we measured proton induced $^{45}Ca^{2+}$ uptake by HEK293 cells

transiently expressing either wild type rat TRPV1 or the T550I mutant in the presence of increasing concentrations of Ab-156H (Fig.3). As expected, control rabbit polyclonal antibodies did not affect proton activation of either wild type rat TRPV1 or the T550I mutant. AMG9810, a small molecule antagonist, inhibited only wild type rat TRPV1 (IC_{50} value: 265.8 $\pm$ 118.3 nM), but not the T500I mutant (IC_{50} value: >40,000 nM), in agreement with predicted loss of AMG9810 binding to the T550I mutant. In contrast, Ab-156H inhibited proton activation of both wild type rat TRPV1 and its T550I mutant with IC_{50} values of 23.9 $\pm$ 4.9 nM and 52.4 $\pm$ 7.6 nM, respectively, demonstrating that disruption of vanilloid binding pocket conformation does not affect Ab-156H binding or antagonism of rat TRPV1.

Rabbit monoclonal antibodies to the human TRPV1 pre-pore peptide bind to TRPV1 but are ineffective at blocking channel activation

Because rabbit polyclonal antibodies to the rat TRPV1 pre-pore loop act as antagonists, we sought to generate monoclonal antagonist antibodies to human TRPV1. We utilized the entire pre-pore region of human TRPV1 (Thr^{598}LIEDGKND SLPSESTSH RWRGPACRP PDSSYNSLY $STCys^{636}$) as an antigen to generate antibodies. First, we evaluated pre-immune and immune serum from the rabbit immunized with the Thr^{598}-Cys^{636} peptide in proton- and capsaicin-induced $^{45}Ca^{2+}$ uptake assays with or without either antigen peptide (Thr^{598}-Cys^{636}) or an unrelated peptide (Fig.4). Pre-immune serum combined with 10 μM of either antigen peptide or an unrelated peptide did not significantly inhibit either pH 5 or capsaicin activation of human TRPV1 (Fig.4A and B). However, immune serum to Thr^{598}-Cys^{636} peptide combined with 10 μM of an unrelated peptide showed complete inhibition of both pH 5 and capsaicin activation. This inhibition was prevented by the antigen peptide (Thr^{598}-Cys^{636}) in a concentration-dependent manner (Fig.4A and B), indicating specific antagonism of TRPV1 activation by the antiserum.

Since rabbit polyclonal antiserum to the human TRPV1 pre-pore region (Thr^{598}-Cys^{636}) showed inhibition of TRPV1 activation, we generated monoclonal antibodies to this pre-pore region following published protocols (Spieker-Polet et al., 1995) through a contract service provided by Epitomics Inc., (Burlingame, CA). Conditioned media were generated using immortalized

clonal cell lines and purified monoclonal IgGs were evaluated in a whole cell ELISA for specific binding to human TRPV1 (Fig.5A). Both the conditioned media and the purified IgGs gave positive signals in whole cell ELISA with CHO cells expressing human TRPV1 but not with parental CHO cells (Fig.5A). Twenty-six purified monoclonal IgGs were identified as specific binders to cells expressing human TRPV1 and were evaluated for antagonism of proton and capsaicin activation in $^{45}Ca^{2+}$ uptake assays.

None of the twenty-six rabbit monoclonal IgGs that are selective binders of human TRPV1 blocked either proton or capsaicin activation in $^{45}Ca^{2+}$ uptake assays (Fig.5B and C respectively). The concentration range of the purified antibodies used in $^{45}Ca^{2+}$ assay was 88.5-266.8 μg/ml. A small molecule antagonist, AMG9810, was used as a positive control in the assay (IC_{50} values at proton and capsaicin activation were 70.60 $\pm$ 7.43 nM and 19.0 $\pm$ 2.7 nM, respectively). We also generated a number of mouse and fully human monoclonal antibodies to the pre-pore loop region of human TRPV1. The majority of these monoclonal antibodies showed selective binding to CHO cells expressing TRPV1 (but not to parental CHO cells); however none were able to block either proton or capsaicin activation of TRPV1 (data not shown).

Discussion

By utilizing agonist-induced $^{45}Ca^{2+}$ uptake assays and CHO cells stably expressing TRPV1, we have characterized a rabbit polyclonal antibody (AB-156H) that was generated against the rat TRPV1 pre-pore region as a novel TRPV1 antagonist. Ab-156H acts as a full antagonist of proton (pH 5 and 5.5) activation, and as a partial antagonist of capsaicin, pH 6 potentiated chemical ligands (capsaicin and anandamide) and heat activation of rat TRPV1, suggesting that Ab-156H partially stabilizes the channel conformation in the closed state. Small molecule antagonists that interact at the capsaicin-binding pocket allosterically block proton activation that occurs through residues in the pre-pore loop. Polyclonal antibody Ab-156H that binds pre-pore region of TRPV1 is allosteric to the capsaicin-binding pocket and hence allosterically blocks capsaicin activation. In agreement with this, Ab-156H antagonism of rat TRPV1 is not affected by disruption of the capsaicin-binding pocket conformation as demonstrated by equal potency of Ab-156H at wild type (capsaicin-sensitive) and the T550I mutant (capsaicin-insensitive) of rat TRPV1. We have previously shown that small molecule TRPV1 antagonists that block all modes of activation appear to lock the channel conformation in the closed state by interacting at the capsaicin-binding pocket (Gavva et al., 2005b) and the current studies with polyclonal antagonist antibody (Ab-156H) demonstrate that the channel conformation can be stabilized partially in the closed state by binding to a region out side of the capsaicin-binding pocket.

Since Ab-156H was more potent at blocking proton activation than activation by chemical ligands or heat, it is possible that Ab-156H inhibition of proton activation occurs by blocking protonation of the residues responsible for activation in addition to locking or stabilizing the channel in the closed state. We hypothesized that if Ab-156H inhibition of protonation occurs simply by occluding the residues from protonation, it would not block capsaicin or anandamide activation. Since Ab-156H acted as a partial antagonist of capsaicin, anandamide and heat activation of TRPV1, we propose that Ab-156H partially locks or stabilizes the channel conformation in the closed or non-conducting state. Since Ab-156H binds to the pre-pore region in the vicinity of the pore, we cannot completely exclude the possibility that Ab-156H acts as a pore blocker of TRPV1.

We previously postulated that TRPV1 antagonists acting through the capsaicin-binding pocket fall into two distinct categories (Gavva et al., 2005c); class A compounds block channel activation both by capsaicin and protons, whereas class B compounds selectively abolish capsaicin activation. The results presented here indicate that the rabbit anti-rat TRPV1 polyclonal antibody (Ab-156H) binding at a site outside of the capsaicin-binding pocket, acts as a full antagonist of proton activation and a partial antagonist of capsaicin, anandamide and heat activation, thus representing a class A antagonist with an unusual profile. This indicates the complexity of mechanisms for antagonism of TRPV1 channel.

Polyclonal antibodies that antagonize the function of ion channels and G-protein coupled receptors reported in the literature include antibodies to the pre-pore regions of Kv1.2, Kv3.1 (Zhou et al., 1998), TRP channels (TRPC1, TRPC6; Xu et al, 2005), antibodies to the second extracellular loop of the 5-Hydroxytryptamine 4 receptor (Salle et al., 2001), and antibodies to the N-terminal peptide region of Protease-activated receptor 2 (Kelso et al, 2005). Monoclonal antibodies to the second extracellular loop of M2 muscarinic receptor were reported to act as partial agonists (Elies et al., 1998). Interestingly, antagonist antibodies generated against Kv1.2, Kv3.1, TRPC1, TRPC6, all have been reported to block 50-60% of channel activation. Since Ab-156H blocks >75% proton activation, it is possible that Ab-156H blocks the channel activation both by partially stabilizing the channel conformation in the closed or non-conducting state and also by directly blocking protonation as well. In agreement with antibodies to ion channels acting as modulators of function, it has been found that antisera from patients with Lambert-Eaton Myasthenic Syndrome inhibit calcium channel currents (Flink and Atchison, 2003 and references therein). However, there are no reports in the literature indicating the existence of autoantibodies to TRPV1 that can cause insensitivity or reduced sensitivity to inflammatory or neuropathic pain.

It is noteworthy that almost all TRPV1 antagonists reported thus far seem to bind at the capsaicin site and thus are competitive antagonists of capsaicin activation. Compounds that block TRPV1 activation via interaction at a site outside of the capsaicin-binding pocket include arginine-rich peptides such as R4W2 (Planells-Cases et al, 2002; Himmel et al, 2002), trialkylglycine-based compounds such as DD161515 and DD191515 (Garcia-Martinez et al., 2002) and acylpolyamine

toxins derived from funnel web spider venom (Kitaguchi and Swartz, 2005). *In vivo* efficacy of peptide, antibody and toxin blockers of TRPV1 has not been demonstrated in models of pain except for a report by Kamei et al (2001) that polyclonal antibodies to TRPV1 (purchased from Neuromics, Bloomington, MN) attenuated thermal hyperalgesia in a diabetic pain model in mice. However, no information is available regarding the antigen peptide or potency at blocking different modes of TRPV1 activation for this antibody. Since the two TRPV1 polyclonal antibodies sold by Neuromics were generated against either an intracellular N- or C-terminus regions of TRPV1, it is not clear how these antibodies acted as antagonists *in vivo*.

To evaluate the feasibility of generating monoclonal antagonist antibodies to TRPV1, we used the entire pre-pore region of human TRPV1 as an antigen in two species, rabbits and mice. Monoclonal antibodies generated in both rabbits and mice selectively bound to TRPV1 expressing cells, however, they did not block channel activation by either protons or capsaicin, suggesting that monoclonal antibodies were not able to lock or stabilize the channel conformation in the closed or non-conducting state. Studies with Ab-156H suggest that polyclonal antibodies may stabilize or lock the channel conformation by binding to the entire 23 amino acid, pre-pore region (Glu^{600}-Pro^{623}) of rat TRPV1, resulting in partial to full antagonism of activation by different stimuli. Monoclonal antibodies may only bind smaller epitope(s) of the 39 amino acid pre-pore region of human TRPV1 (Thr^{598}-Cys^{636}), and hence may not lock or stabilize the channel in the closed state and are ineffective against all modes of activation. Although it is recognized that antibody binding and function can be influenced by factors such as temperature, pH and salt concentration (Lipman et al, 2005), it is unlikely that this can explain the differences between monoclonal and polyclonal antibodies as these bound to the channel but blocked activation differentially in the functional assays. Since no rabbit, mouse or fully human monoclonal antibodies generated against the pre-pore region of human TRPV1 (Thr^{598}-Cys^{636}) were effective at blocking channel activation, we hypothesize that it may not be possible to lock the channel conformation through high affinity binders to small epitopes in this region. Interestingly, our attempts to mix several monoclonal antibodies to mimic a polyclonal antibody did not result in antagonism of either proton or capsaicin activation of human TRPV1 (data not shown). Since it is not known to which epitope each monoclonal antibody binds within the pre-pore region (Thr^{598} – Cys^{636}), we are not sure if a mix of monoclonal antibodies would represent

a true polyclonal antibody. No monoclonal antagonist antibodies to ion channels have been identified to date. It remains to be seen if monoclonal antibodies targeting pre-pore regions in ion channels can act as antagonists.

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Figure Legends

Figure 1. A) The amino acid sequence alignment of the human, cynomolgous monkey, rat, rabbit, mouse, and guinea pig TRPV1 pre-pore loop region. A Glu^{600}-Pro^{623} peptide was used to generate rabbit polyclonal antibody Ab-156H against rat TRPV1 is underlined. A Thr^{598}-Cys^{636} peptide was used to generate rabbit monoclonal antibodies against human TRPV1. B) Immunochemical characterization of rabbit polyclonal antibody; affinity-purified anti-ratTRPV1 polyclonal antibody Ab-156H (approximately 1 μg/ml), but not a control rabbit polyclonal antibody (Rb-IgG) detects rat TRPV1 in transiently expressed in HEK293 cells; Glu^{600}-Pro^{623} peptide (2.6 μM) blocks Ab-156H binding to rat TRPV1.

Figure 2. Ab-156H inhibits proton (A,B), chemical ligand (C), and heat (D) 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 with increasing concentrations of Ab-156H antibody followed by addition of agonist. To test the ability of antigen peptide to compete with Ab-156H antagonism of proton activation (B), Glu^{600}-Pro^{623} peptide (20 μM) and Ab-156H (2.7 μM) were pre-incubated prior to pH 5-induced activation of rat TRPV1. Ab-156H inhibits non-potentiated and pH 6.0 potentiated capsaicin activation (E). The % inhibition by Ab-156H compared to Rb-IgG is shown above the bars in the graph (E). A control rabbit polyclonal antibody (Rb-IgG) does not block pH or capsaicin activation in D and E. Each point in the graph is an average $\pm$ SD. of an experiment conducted in triplicate.

Figure 3. Ab-156H inhibits proton activation of both wild type rat TRPV1 (A) and capsaicin-insensitive mutant rat TRPV1-T550I (B). HEK293 cells transiently expressing wild type rat TRPV1 or the T550I mutant were used in proton induced $^{45}Ca^{2+}$ uptake assay. Cells were pre-incubated with increasing concentrations of Ab-156H, control rabbit polyclonal antibody (Rb IgG), or a small molecule antagonist (AMG9810). Each point in the graph is an average $\pm$ SD of an experiment conducted in triplicate.

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Figure 4. Rabbit polyclonal serum raised against a human TRPV1 pre-pore peptide (Thr^{598}-Cys^{636}) inhibits proton (A) and capsaicin (B) activation. Pre-immune serum (PIS) and serum from rabbits immunized with Thr^{598}-Cys^{636} peptide (IS) were pre-incubated with cells expressing human TRPV1 (as described in *Methods and Materials* section) in the presence of increasing concentrations of antigen (Ag pep) or irrelevant peptides (Ir pep). $^{45}Ca^{2+}$ uptake was measured as described in *Methods and Materials* section. Please note that Ir pep (10 μM) combined with PIS, or Ag pep (10 μM) combined with PIS, did not affect either proton or capsaicin activation, whereas Ag pep, but not Ir pep, prevented inhibition of both proton and capsaicin activation by IS in a concentration dependent manner.

Figure 5. Immunochemical characterization of rabbit anti-humanTRPV1 monoclonal antibodies (A). Monoclonal antibodies that selectively bind to human TRPV1 expressing cells were identified using whole cell ELISA. Note that conditioned media and purified monoclonal IgG gave a positive signal in whole cell ELISA with TRPV1 expressing cells but not with parental CHO cells. Purified monoclonal antibodies that are selective TRPV1 binders showed no inhibition of either proton (B) or capsaicin (C) activation of human TRPV1. TRPV1 expressing cells were incubated with increasing concentrations of purified monoclonal IgG or the small molecule antagonist, AMG9810 as indicated, followed by the addition of agonists for 2 minutes. Each point in the graph is an average $\pm$ SD of an experiment conducted in triplicate.

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Figure 1

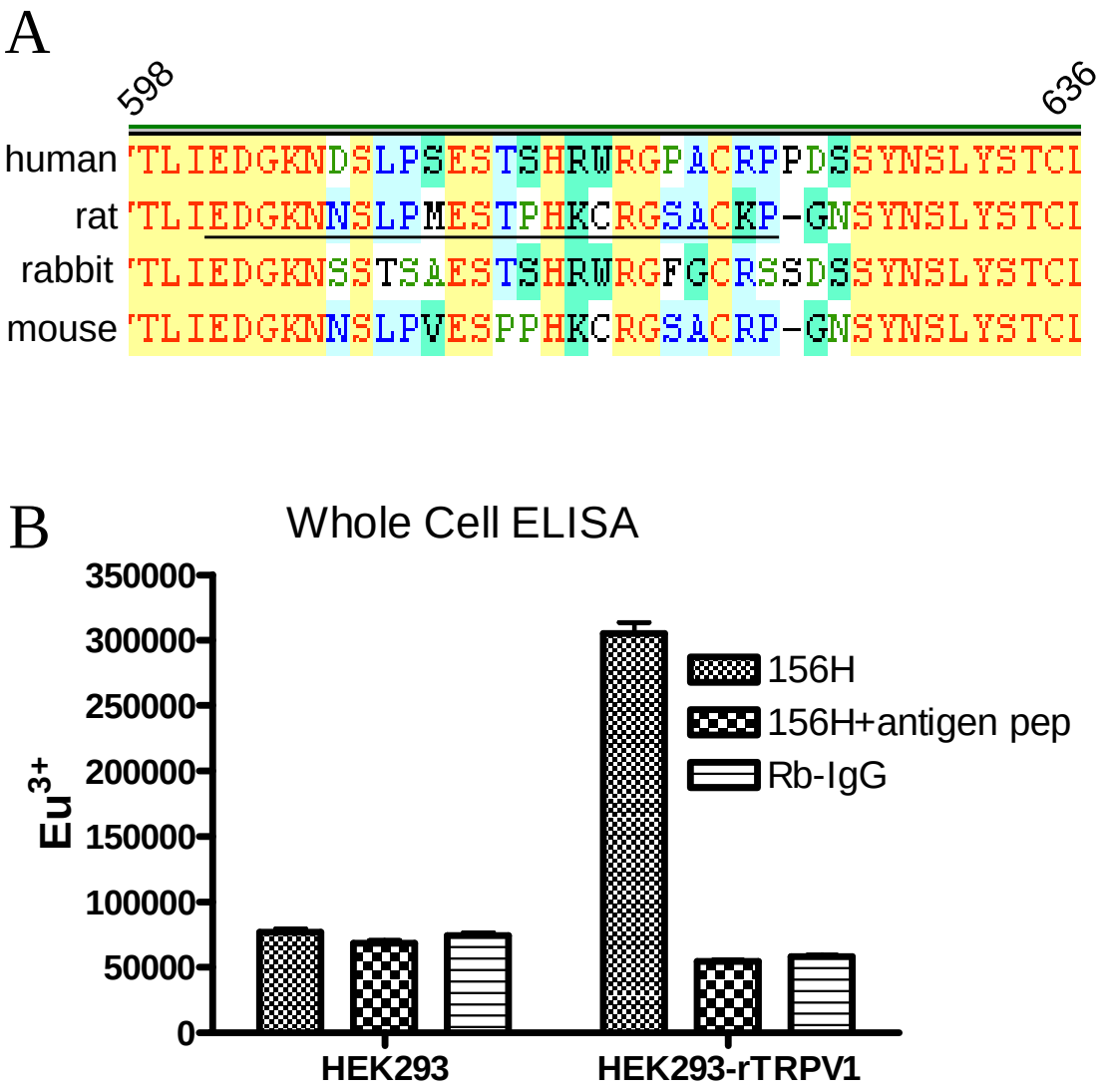

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Figure 2

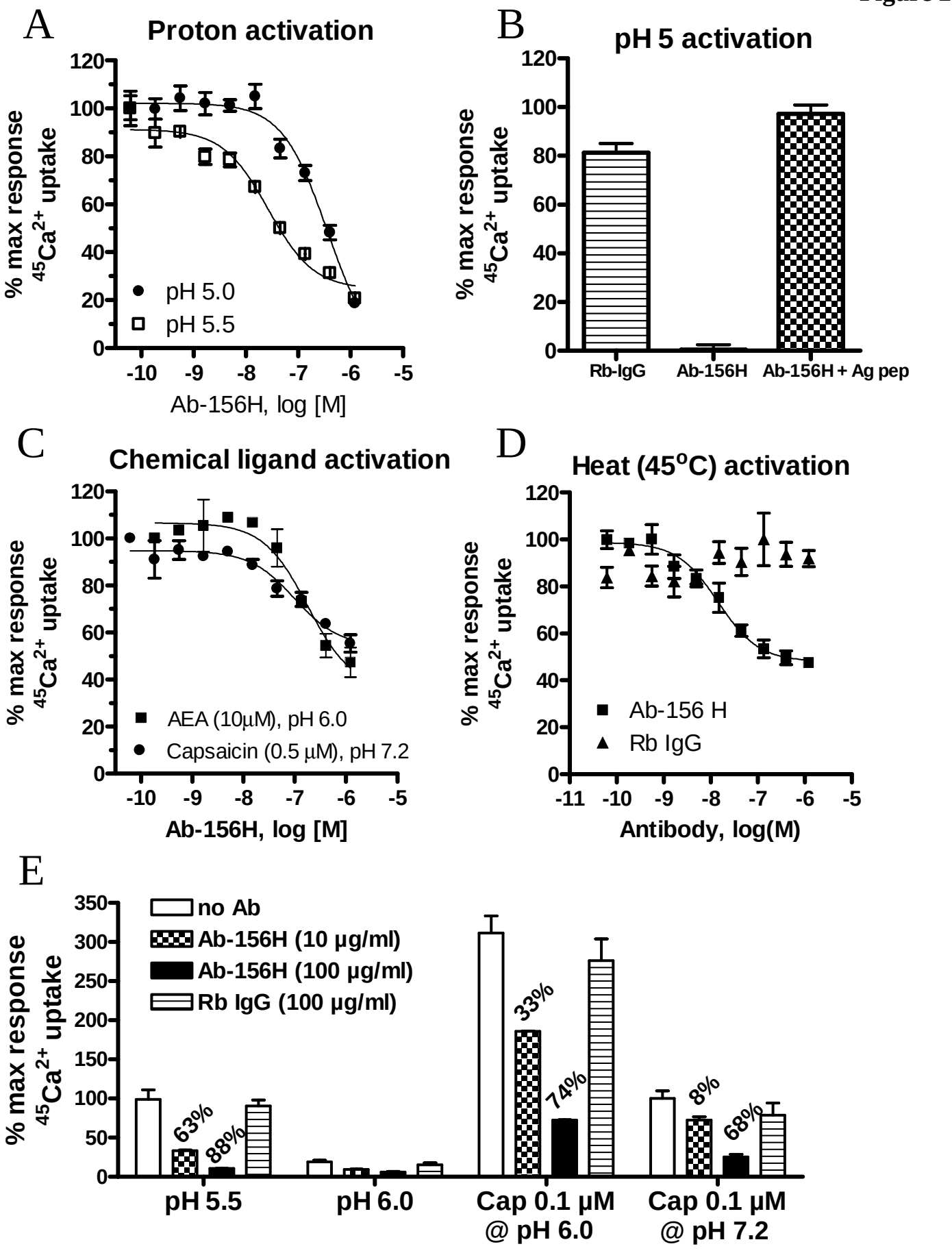

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Figure 3

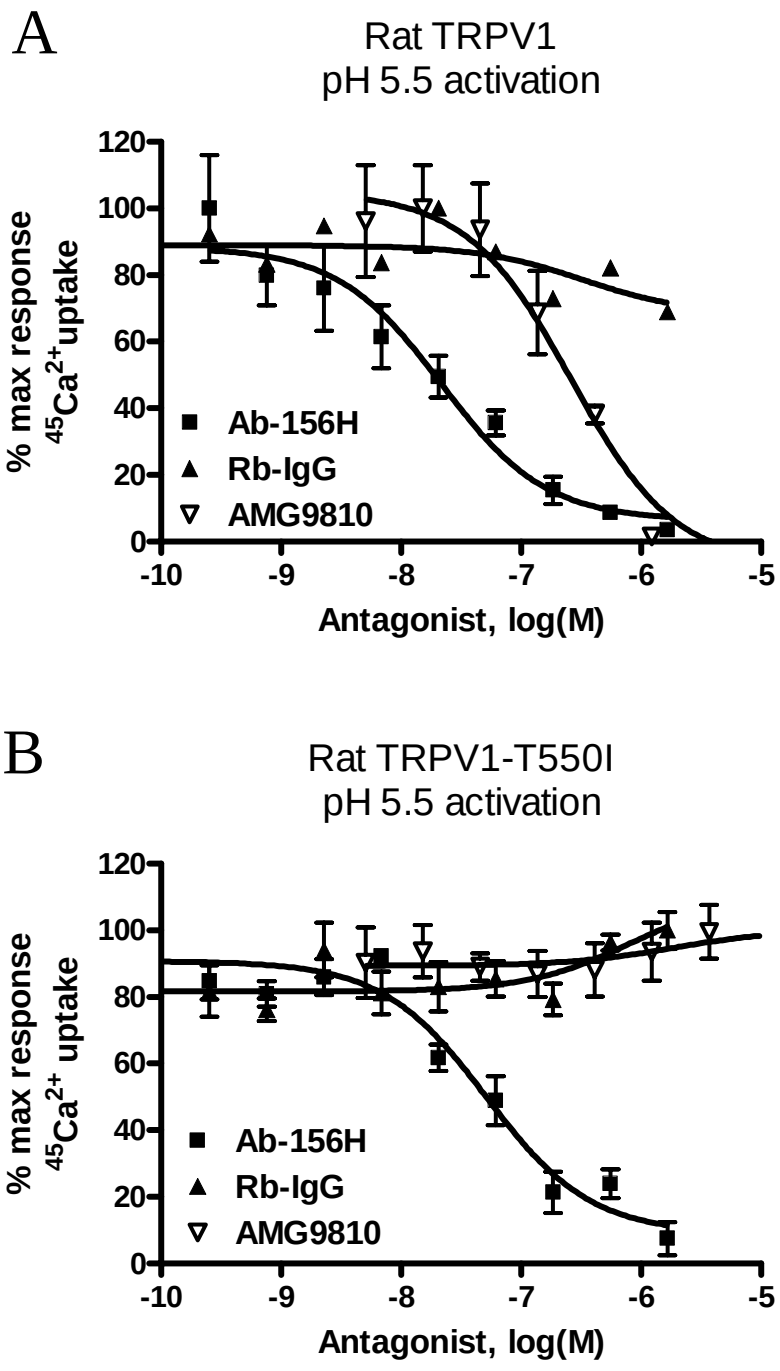

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Figure 4

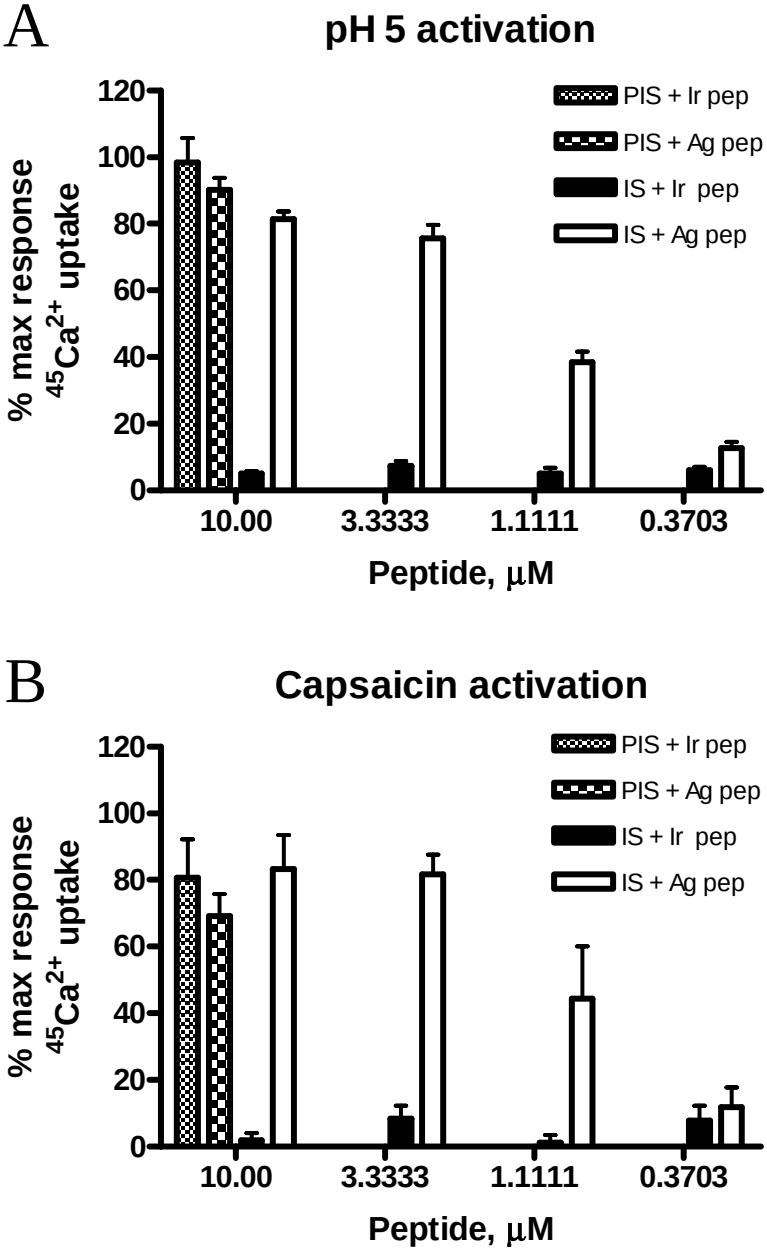

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Figure 5