BJP British Journal of Pharmacology

DOI:10.1111/bph.12005
www.brjpharmacol.org

RESEARCH PAPER

Altered pharmacology of native rodent spinal cord TRPV1 after phosphorylation

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Keywords
TRPV1; CGRP release; phosphorylation; spinal cord

Received
18 April 2012
Revised
6 September 2012
Accepted
14 September 2012

BACKGROUND AND PURPOSE

Evidence suggests that phosphorylation of TRPV1 is an important component underlying its aberrant activation in pathological pain states. To date, the detailed pharmacology of diverse TRPV1 receptor agonists and antagonists has yet to be reported for native TRPV1 under phosphorylating conditions. Our goal was to optimize a relatively high-throughput methodology to allow pharmacological characterization of the native TRPV1 receptor using a spinal cord neuropeptide release assay under naive and phosphorylating states.

EXPERIMENTAL APPROACH

Herein, we describe characterization of rodent TRPV1 by measurement of CGRP release from acutely isolated lumbar (L1-L6) spinal cord using a 96-well technique that combines use of native, adult tissue with quantitation of CGRP release by ELISA.

KEY RESULTS

We have studied a diverse panel of TRPV1 agonists and antagonists under basal and phosphorylating conditions. We show that TRPV1-mediated CGRP release is evoked, in a temperature-dependent manner, by a PKC activator, phorbol 12,13-dibutyrate (PDBu); and that treatment with PDBu increases the potency and efficacy of known TRPV1 chemical agonists, in an agonist-specific manner. We also show that the pharmacological profile of diverse TRPV1 antagonists is dependent on whether the stimulus is PDBu or capsaicin. Of note, HPPB was identified as an antagonist of capsaicin-evoked, but a potentiator of PDBu-evoked, CGRP release.

CONCLUSIONS AND IMPLICATIONS

Our findings indicate that both TRPV1 agonist and antagonist profiles can be differentially altered by PKC activation. These findings may offer new insights for targeting TRPV1 in pain states.

Abbreviations

BCTC, *N*-(4-*t*-butylphenyl)-4-(3-chloropyridin-2-yl) tetrahydropryazine-1(2*H*)-carboxamide; CGRP, calcitonin-gene-related peptide; HPPB, *N*-(3-hydroxyphenyl0-4-phenyl-benzamide; NADA, *N*-arachidonoyl dopamine; OLDA, *N*-oleoyldopamine; PDBu, phorbol 12,13-dibutyrate; RTX, resiniferatoxin; TRPV1, transient receptor potential vanilloid subtype 1

Introduction

Transient receptor potential vanilloid subtype 1 (TRPV1) is a Ca^{2+}-permeable cation channel that acts as a polymodal receptor responding to noxious chemical and physical stimuli, including capsaicin, heat (>43°C) and acidic pH ≤5.9 (Caterina *et al.*, 1997; Tominaga *et al.*, 1998). Additionally, TRPV1 is activated by endogenous lipid mediators such as anandamide and *N*-arachidonyldopamine (NADA) and lipoxegenase products including 12-HPETE and leukotriene B4

(Zygmunt *et al.*, 1999; Hwang *et al.*, 2000; Huang *et al.*, 2002; Huang and Walker, 2006). TRPV1 is also sensitized by protein kinases, including PKC and PKA (Premkumar and Ahern, 2000; De Petrocellis *et al.*, 2001; Vellani *et al.*, 2001; Bhave *et al.*, 2002; 2003; Crandall *et al.*, 2002; Premkumar *et al.*, 2004).

Consistent with a role in nociception, TRPV1 is dominantly expressed in a subset of primary afferent fibres, with activation of TRPV1 eliciting release of neuropeptides (e.g. CGRP, Substance P) and neurotransmitters (e.g. glutamate) from the peripheral and central terminals (Wardle *et al.*, 1997; Kanai *et al.*, 2005; Schicho *et al.*, 2005; Lappin *et al.*, 2006; Puttfarcken *et al.*, 2010).

Following tissue injury, there is local inflammation caused by the release of endogenous growth factors, protons and neuropeptides. Inflammatory mediators such as ATP, trypsin, bradykinin, PGE_2 and nerve growth factor act at their cognate G-protein-coupled receptors/tyrosine kinase receptors to activate signalling cascades leading to generation of lipid metabolites and activation of protein kinases. Integration of these diverse signals leads to a dynamic reduction in the threshold of TRPV1 activation (Ma and Quirion, 2007; Szallasi *et al.*, 2007). This molecular integration likely underlies the aberrant activation of TRPV1 seen under *in vivo* pathological injury or inflammatory states (Kanai *et al.*, 2005; Schicho *et al.*, 2005; Lappin *et al.*, 2006; Puttfarcken *et al.*, 2010). In line with this, thermal hyperalgesia under inflammatory conditions is strongly attenuated in TRPV1 knock-out mice (Caterina *et al.*, 2000; Davis *et al.*, 2000) and a number of highly selective TRPV1 antagonists are efficacious across multiple pre-clinical models of pain (Pomonis *et al.*, 2003; Honore *et al.*, 2005; Kanai *et al.*, 2007; Lehto *et al.*, 2008; Puttfarcken *et al.*, 2010).

In addition to TRPV1 antagonists, TRPV1 agonists are also of significant interest for alleviating pain in humans. TRPV1 agonists, including capsaicin and resiniferatoxin are utilized clinically, or are being clinically assessed for efficacy in treating some conditions of chronic pain; however, the route of administration may limit efficacy and broad applicability (Iadarola and Mannes, 2011). For both TRPV1 agonist and antagonist approaches, adverse effects have significantly hindered clinical progression. Hyperthermia and impaired noxious heat sensation are key concerns for TRPV1 antagonist therapies, whilst systemic toxicity is a limiting issue for agonist approaches (Wong and Gavva, 2009; Trevisani and Szallasi, 2011). Recent work has focused on identifying safer ways to target TRPV1, such as stimulus-mode specific antagonists devoid of hyperthermia (Garami *et al.*, 2010; Watabiki *et al.*, 2010), allosteric modulators (Kaszas *et al.*, 2012) or ligands that selective target pain state TRPV1. For the latter, evidence is accumulating that phosphorylated TRPV1 is highly relevant to pain states.

In preclinical animal models, increased phosphorylation of TRPV1 appears to be a feature common to diverse pain states. In particular, a growing body of data implicates phosphorylation by PKC/PKCε as a key step in TRPV1 sensitization under pathological conditions. PKCε is activated downstream of numerous inflammatory mediators (Cesare *et al.*, 1999; Amadesi *et al.*, 2006; Plant *et al.*, 2006; Zhang *et al.*, 2007; Pan *et al.*, 2010) and directly phosphorylates and sensitizes TRPV1 (Numazaki *et al.*, 2002; Mandadi *et al.*, 2006). PKC-induced phosphorylation underlies the reduction in heat threshold required for TRPV1 activation in the presence of inflammatory mediators such as bradykinin and ATP and is also reported to reverse TRPV1 desensitization and up-regulate TRPV1 channel expression (Premkumar and Ahern, 2000; Chuang *et al.*, 2001; Mandadi *et al.*, 2004; Morenilla-Palao *et al.*, 2004; Sculptoreanu *et al.*, 2008). PKC-induced TRPV1 sensitization is associated with pain states, including diabetic neuropathy (Hong and Wiley, 2005), bladder cystitis (Sculptoreanu *et al.*, 2005) and bone cancer (Pan *et al.*, 2010), with altered phosphorylation and/or function reported in dorsal root ganglion from these animals. *In vivo*, pain behaviours induced by injection of a PKC activator are absent in TRPV1 knock-out mice (Bolcskei *et al.*, 2005); conversely, PKCε has been shown to be a requirement for capsaicin and acid induced pain behaviours (Khasar *et al.*, 1999; Jung *et al.*, 2004; Srinivasan *et al.*, 2008).

If, as these findings indicate, TRPV1 in pathological pain conditions exists in a hyper-phosphorylated state, it would be important to assess TRPV1 agonist and antagonist profiles under these conditions. Whilst there are several studies in recombinant systems on phosphorylated TRPV1 (Wang *et al.*, 2003; El-Kouhen *et al.*, 2005; Swanson *et al.*, 2005; Pearce *et al.*, 2008), the pharmacological characterization performed to date in native systems is limited (Fischer and Reeh, 2007; Sikand and Premkumar, 2007).

We have studied TRPV1 evoked CGRP release from acutely isolated rat lumbar spinal cord in response to a structurally diverse set of TRPV1 agonists and antagonists under basal and phosphorylating conditions. Phosphorylation by PKC increased the potency of known TRPV1 chemical agonists, in an agonist specific manner, in addition to significantly altering the inhibitory profile of structurally diverse TRPV1 antagonists. The data provide new insights into the pharmacology of native TRPV1 under phosphorylating conditions, which may be highly relevant to pain states.

Methods

Materials

Standard buffer components were of analytical grade and were purchased from Fisher Scientific (Loughborough, UK). Commercially available compounds were purchased from Sigma Aldrich (Gillingham, UK) or Tocris-Cookson (Bristol, UK). TRPV1 antagonists, with the exception of capsazepine, HPPB, SB366791, and AMG9810, were obtained from the chemical synthesis laboratory (Eli Lilly & Company, Indianapolis, IN). HPPB was from Asinex (Moscow, Russia). Ninety-six-well multi-screen filter plates were from Millipore Inc. (Abingdon, UK). CGRP release ELISA kits were obtained from SPI BIO (Montigny le Bretonneux, France). TRPV1 knock-out mice were from Jackson Laboratories (Sacramento, CA, USA). C57BL/6 wild-type mice and Lister-Hooded rats were obtained from Charles River (Margate, UK). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). All animal procedures and experiments were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and conformed to institutional regulations at Eli Lilly & Company Ltd.

Methodology for 96-well CGRP release assay

Experiments were conducted in Krebs buffer composed of (in mM) 118 NaCl, 2.4 KCl, 2.4 $CaCl_2.2H_2O$, 1.2 $MgSO_4.7H_2O$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 10 Glucose, 0.1% DMSO (v/v) ±10 μM thiorphan (to prevent CGRP breakdown by endogenous enkephalinases). Buffer was gassed with 5% CO_2/95% O_2 for 1 h to pH 7.4 and was kept at 37°C using a heated water bath. For Ca^{2+}-free experiments, $CaCl_2.2H_2O$ was omitted from the buffer and 1 mM EGTA added. $MgSO_4{\cdot}7H_2O$ concentration was increased to a concentration of 3.6 mM in order to maintain osmolarity. Compound stocks were prepared in DMSO and then diluted to the required concentrations in Krebs buffer containing thiorphan.

Male, adult (250–350 g), Lister-Hooded rats or C57BL/6 mice (wild type or TRPV1 knock-out) were killed by exposure to a rising concentration of CO_2, followed by cervical dislocation. The lumbar portion (L1–L6) of the spinal cord and vertebrae was rapidly dissected out and the whole cord (including lumbar enlargement) obtained by hydraulic extrusion. This was immediately placed into ice-cold buffer in the absence of thiorphan and weighed (180–220 mg or 20–25 mg wet weight/cord for rat or mouse, respectively) and homogenized using 20 strokes of a glass-Teflon hand homogenizer. The crude homogenate was diluted to an optimal 4 mg mL^{-1} (rat or mouse) wet weight with Krebs buffer in the absence of thiorphan and spun for 1 min at 1500 rpm (363× *g*) in a bench-top centrifuge. It was determined that tissue from one rat or eight mice was sufficient for four 96-well plates and could be diluted further with only slight loss of signal. Supernatant containing the synaptosomal preparation was carefully decanted and 100 μL (0.2–0.3 mg mL^{-1} protein) placed in each well of a 96-well filter plate. Use of these filter plates allowed stimulating ligands to be applied and resulting CGRP release removed without disturbance to the synaptosomes on the filters of the plate. Plates were incubated at 37°C for 30 min prior to compound addition. After the incubation period, filter plates were removed, and the buffer filtered to waste using a vacuum manifold. The contents of the basal plate were added (100 μL per well), and the filter plate was incubated for 10 min at 37°C. The contents of the filter plate were then vacuumed to waste, and the contents of the stimulation plate were added (100 μL per well) and incubated for a further 10 min at 37°C.

At the end of the 10 min stimulation period, the contents of the filter plate were transferred to the ELISA immunoplate by centrifugation at 200× *g*. Tracer was then added, and the immunoplate was covered with plastic film and incubated at 4°C for 16–20 h. The following day the immunoplate was washed and Ellman's reagent added, and the plate was left at room temperature to develop in the dark for 45 min. Limit of detection for the ELISA was approximately 3 pg mL^{-1} of CGRP. After the development time, the absorbance of each well was measured at 405 nm using a 96-well spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA).

Data analysis

Raw absorbance values were imported into SigmaPlot v10, and the amount of CGRP released reported as a percentage of the capsaicin (0.3 μM) or 40 mM KCl control minus the basal release (the amount of CGRP released upon addition of buffer or buffer plus 10 nM PDBu). Graphs of percentage release were drawn using SigmaPlot, and curves were fitted using the four-parameter Hill equation. Where stated, *n* refers to the number of independent experiments performed using spinal cord tissue from different animals. Significant differences in curve-fitting parameters (EC_{50}/IC_{50}/% efficacy/% inhibition) were calculated in GraphPad Prism 5 (La Jolla, CA, USA) using the extra sum of squares *F*-test.

Assay characteristics

Statistical assessment of assay performance was performed using capsaicin as the stimulus. Plate uniformity and signal variability assessment were performed over 3 days with *Z'* values (a measure of statistical effect size) ranging between 0.53 to 0.84 for replicates of four wells, meaning robust data generation was possible with a minimum number of experimental repeats. There was no evidence of significant drift or edge effects. The 96-well format enabled construction of full concentration response curves and thus comparison of pEC_{50}/IC_{50} and efficacy/inhibition values for the compounds tested.

Measurement of intracellular Ca^{2+}

Recombinant CHO stably expressing rat TRPV1 were cultured in DMEM : F-12 Ham (1:3) plus 10% (v/v) FBS, 0.02 M HEPES, penicillin/streptomycin/glutamate (10 000 units, 10 000 μg and 29.2 mg mL^{-1}, respectively) and 1.25 μg mL^{-1} Geneticin G418 sulphate, and incubated in a 37°C humidified incubator with 5% CO_2 in air. They were plated into black clear-bottomed 96-well plates 24 h prior to experiments, at a concentration of 40 K per well.

Receptor-mediated changes in intracellular Ca^{2+} concentration were determined using a Ca^{2+}-sensitive fluorescent dye, Fluo3-AM (Invitrogen, Paisley, UK) and a fluorometric imaging plate reader with heated stage (FLIPR, Molecular Devices Corporation). All assays were performed using HBSS assay buffer, supplied by Invitrogen (Gibco 14025-050) supplemented with 10 mM HEPES and adjusted to pH 7.2. At the start of the assay, growth media was removed from cell plates by inversion and gentle tapping and replaced with assay buffer containing 10 μM Fluo-3AM/0.05% pluronic F-127. Cell plates were stored in the dark at room temperature for 60 min, to allow dye loading into cells. Subsequently, the dye solution was removed and replaced with assay buffer (at 21°C or 37°C), and cell plates were transferred to the FLIPR for assay. Intracellular Ca^{2+} levels were monitored before and after the addition of compounds (at 21°C or 37°C). Responses were measured as the maximal peak height in relative fluorescent units (RFUs), and the assay window was defined as the maximal response obtained by capsaicin (10 μM)-stimulated wells. All RFU values were corrected for basal fluorescence in the absence of agonist. Graphs of percentage stimulation or inhibition were drawn using SigmaPlot, and curves were fitted using the four-parameter Hill equation. Where stated, *n* refers to the number of independent experiments performed using cells plated on different days. Significant differences in curve-fitting parameters (EC_{50}/IC_{50}/% efficacy/% inhibition) were calculated in GraphPad Prism 5 using the extra sum of squares *F*-test.

Results

Capsaicin and PDBu-induced CGRP release are mediated by TRPV1 activation

The prototypic TRPV1 agonist capsaicin (0.001–1 μM) evoked a concentration-dependent increase in release of CGRP from adult rat spinal cord homogenate. The dose–response curve obtained for capsaicin appeared monophasic with an pEC_{50} of 6.99 ± 0.19 M and a maximal response at 300 nM (Figure 1A). In the absence of extracellular Ca^{2+}, or in the presence of a TRPV1 antagonist, *N*-(4-*t*-butylphenyl)-4-(3-chloropyridin-2-yl) tetrahydropryazine-1(2*H*)-carboxamide (BCTC), capsaicin failed to evoke CGRP release (Figure 1A).

To assess if PKC activation had any effects on basal, or TRPV1 agonist evoked CGRP release, homogenate was exposed to an activator of PKC, phorbol 12,13-dibutyrate (PDBu), for 10 min, followed by TRPV1 agonist addition. Application of PDBu alone was able to elicit CGRP release in a concentration-dependent manner, with an pEC_{50} of 6.77 ± 0.07 M and a maximal efficacy near to that of a maximally effective concentration of capsaicin (94 ± 8%). PDBu-stimulated CGRP release was inhibited by the TRPV1 antagonist BCTC and by removal of extracellular Ca^{2+} (Figure 1B). PDBu-stimulated release was also fully blocked by a PKC inhibitor, Ro-31-8220, whilst a close analogue of PDBu, 4α-PDD, which does not activate PKC, failed to evoke CGRP release (Figure 1C,D). There was a clear temperature-dependent increase in efficacy of PDBu-evoked CGRP release, which was significantly greater at 30°C and 37°C compared with 21°C [Figure 1E; 49 ± 2% efficacy at 21°C vs. 67 ± 4% at 30°C ($P < 0.01$) vs. 89 ± 5% at 37°C ($P < 0.05$)]. Additional experiments conducted in a recombinant system gave results mirroring those obtained in the native release experiments. In CHO cells stably expressing rat TRPV1, PDBu caused a concentration dependent increase in $[Ca^{2+}]_i$, as measured using fluo-3 and a FLIPR, which was significantly greater at 37°C versus room temperature [65 ± 3% and 24 ± 1%, respectively ($P < 0.01$)], and which was fully blocked by incubation with the TRPV1 antagonist BCTC. The potencies of PDBu to evoke functional responses in the recombinant system (pEC_{50} = 7.03 ± 0.1 M) and of BCTC to block the response (pIC_{50} = 8.81 ± 0.1 M) corresponded closely to values observed in the native preparation (Figure S1).

Release of CGRP was also assessed in spinal cord tissue from wild-type and TRPV1 null-mutant mice (Figure 2). In tissue from wild-type mice, capsaicin and PDBu both evoked CGRP release in a concentration-dependent manner with pEC_{50} values of 7.64 ± 0.23 M and 7.66 ± 0.06 M respectively (Figure 2A). In contrast, in spinal cord tissue from TRPV1 null-mutant mice, neither PDBu nor capsaicin elicited CGRP release above the basal control (Figure 2B).

Sub-maximal PKC activation enhances TRPV1 sensitivity to agonists in an agonist specific manner

To assess the effect of sub-maximal PKC activation on the pharmacological profile of a panel of TRPV1 agonists, responses to agonists were investigated under basal or phosphorylating conditions (Figure 3). In preliminary experiments, 10 nM PDBu was selected as whilst it produced only a small increase in basal CGRP release when applied alone, it significantly sensitized responses to capsaicin (pEC_{50} = 7.57 ± 0.14 M, $P < 0.001$, Figure 3A). Addition of the PKC inhibitor Ro-31-8220 prevented this PDBu-induced increase in TRPV1 agonist potency (pEC_{50} = 7.05 ± 0.05 M; Figure 3A).

The additional TRPV1 agonists tested all evoked CGRP release in a concentration-dependent manner (Figure 3B–H) with a rank order of potency of resiniferatoxin > capsaicin > arvanil > *n*-arachidonoyl dopamine (NADA) > anandamide > *n*-oleoyldopamine (OLDA) > olvanil > 9-HODE. OLDA, anandamide, olvanil, NADA and 9-HODE appeared to be partial agonists with a rank order of efficacy of OLDA > anandamide > olvanil > NADA > 9-HODE (Table 1). Addition of the TRPV1 antagonist BCTC (1 μM) largely inhibited TRPV1 agonist evoked CGRP release to the level of the basal control (Figure 3B–H). In the presence of 10 nM PDBu, increases in potency and efficacy were seen for most TRPV1 agonists (Table 1). However, the PKC-mediated potency shift appeared to be agonist dependent, with potency shifts ranging from 1.1-fold (olvanil) to 8.8-fold (arvinal) and increases in efficacy ranging from 1.1-fold (RTX) to 2.3-fold (anandamide) (Table 1).

Effect of PKA activation on TRPV1 function in rat spinal cord tissue

The effect of PKA activation on basal and stimulated CGRP release was examined using forskolin to stimulate cAMP production and activate PKA. Forskolin, in the presence of 100 μM IBMX to inhibit breakdown of cAMP, evoked a small increase in basal CGRP release that was highest at 100 μM (19 ± 3% of 300 nM capsaicin response) and inhibited by 1 μM BCTC (Figure 4A). Pre-incubation (10 min) of rat spinal cord homogenate with 30 μM forskolin/100 μM IBMX, followed by application of capsaicin induced a small leftward shift in the potency for capsaicin (Figure 4B; pEC_{50} = 7.11 ± 0.04 M vs. 7.36 ± 0.08 M ($P < 0.05$), in the absence and presence of forskolin respectively). Interestingly, a small increase in potency of PDBu was also seen in the presence of forskolin (Figure 4C; pEC_{50} = 6.71 ± 0.16 M vs. 7.06 ± 0.13 M, P = 0.051, in the absence and presence of forskolin respectively).

TRPV1 antagonists can differentially block capsaicin versus PKC stimulated TRPV1

The pharmacology of rat spinal TRPV1 under basal and phosphorylating conditions was further explored using a panel of TRPV1 antagonists (Figure 5–7). TRPV1 antagonists were assessed for their ability to inhibit capsaicin and PDBu-evoked CGRP release (Figure 5–6). All antagonists tested fully inhibited capsaicin-evoked CGRP release (Table 2); however, effects on PDBu-mediated release were more divergent (Tables 2,3).

The majority of antagonists tested (including BCTC, AMG517, A425619, A784168, AMG 49a, AMG9810, JNJ 17203212, SB705498, Neurogen) fully inhibited CGRP release evoked by 300 nM capsaicin or 1 μM PDBu with similar potency values (Figure 5; Table 2; Supporting Information, Figure S2). A correlation plot comparing the potency of all antagonists tested versus capsaicin or PDBu showed a similar rank order of potency and a strong positive correlation (R = 0.91; Supporting Information, Figure S3).

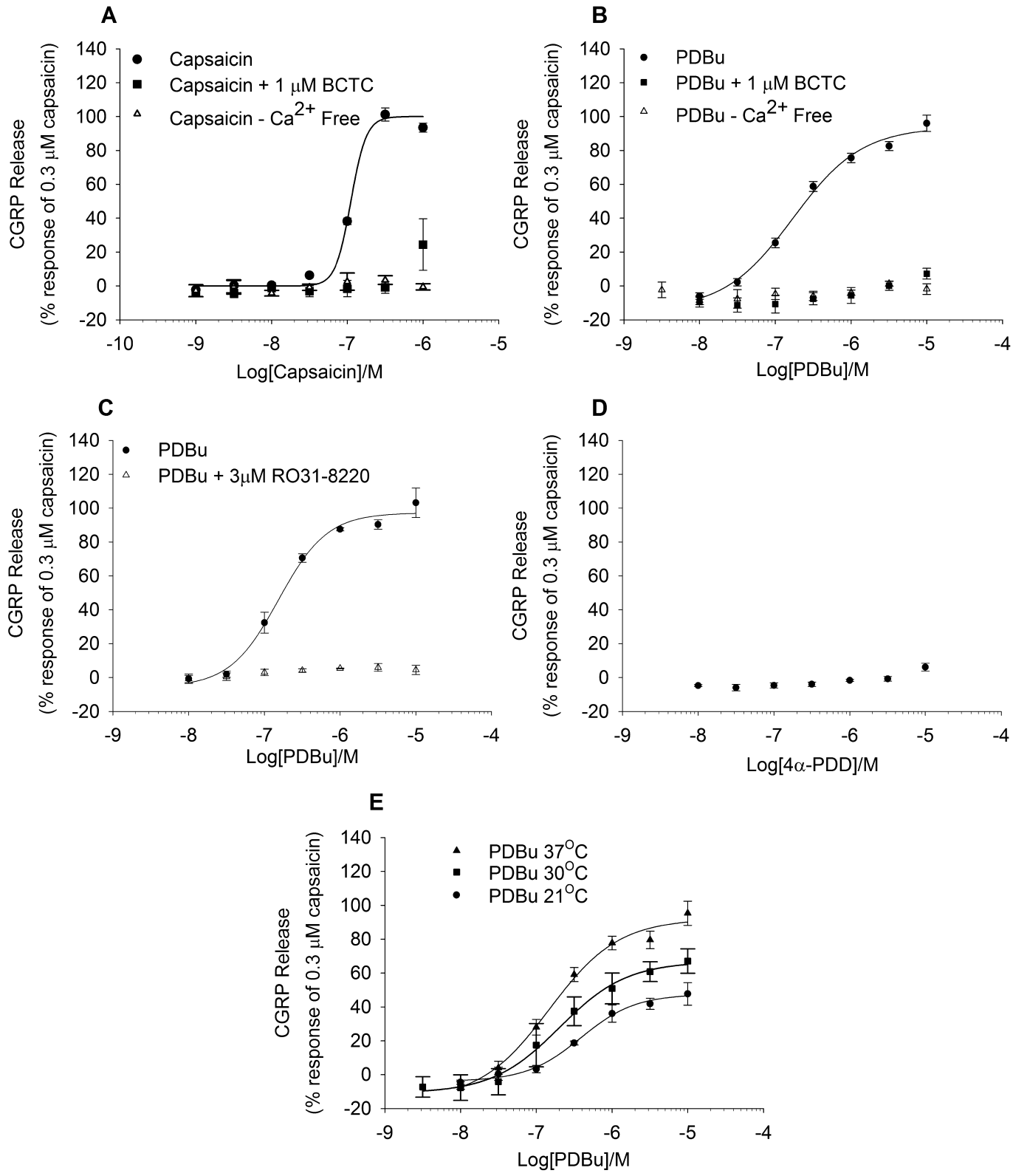

Figure 1

Initial characterization of capsaicin and PDBu evoked CGRP release from rat spinal cord homogenate. (A and B) Concentration–response curves to capsaicin or PDBu were inhibited by the TRPV1 antagonist BCTC and by removal of extracellular Ca^{2+}. (C) Responses to PDBu were inhibited by the PKC inhibitor Ro-31,3220. (D) The structural analogue of PDBu, 4α-PDD did not evoke significant CGRP release above basal. (E) Responses to PDBu were enhanced at 30°C and 37°C as compared with 21°C. Responses were calculated as a percentage of 0.3 μM capsaicin in buffer containing Ca^{2+}. Each data point represents the mean release ± SEM from at least three separate experiments.

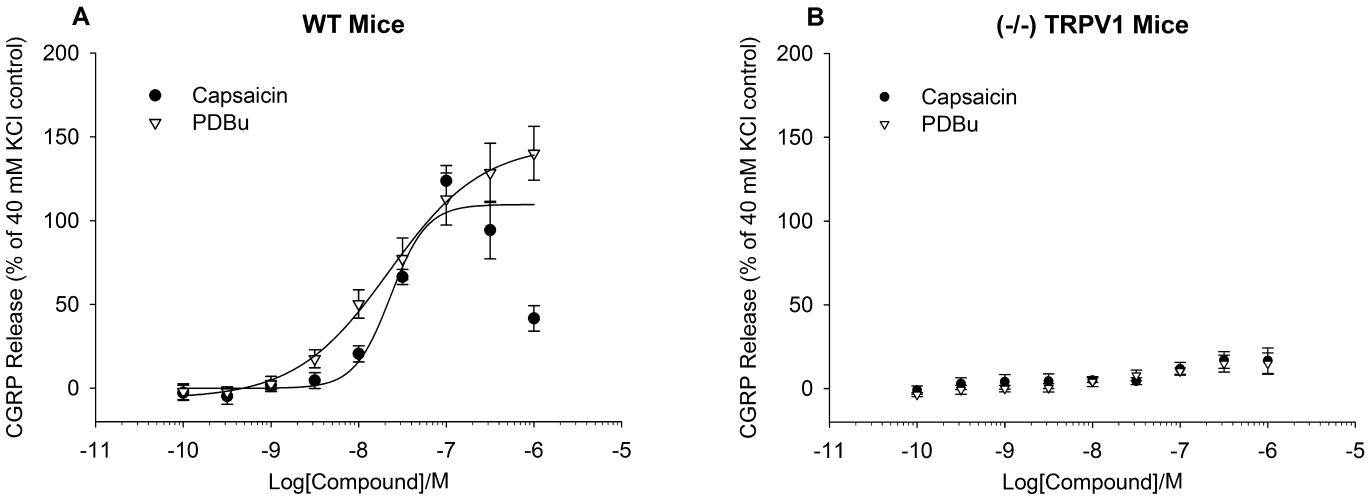

Figure 2

Release of CGRP from mouse spinal cord homogenate. Responses to capsaicin or PDBu were assessed using tissue from either wild-type (A) or TRPV1 knockout mice (B). Responses were calculated as a percent of the response to 40 mM KCl. Each data point represents the mean release ± SEM from three separate experiments.

Table 1

Summary of the effects of TRPV1 agonists on CGRP release from rat spinal cord homogenate in the absence and presence of the PKC activator PDBu

	Agonist alone			Agonist + 10 nM PDBu				
Compound	**pEC_{50} ± SEM (M)**	**Efficacy (%)**	***n***	**pEC_{50} ± SEM (M)**	**Efficacy (%)**	***n***	**Fold shift in EC_{50}**	**Fold increase in efficacy**
Capsaicin	6.99 ± 0.19	98 ± 6	14	7.46 ± 0.14	97 ± 14	7	2.9**	1.3
RTX	8.72 ± 0.06	84 ± 6	9	8.95 ± 0.13	100 ± 14	3	1.7	1.2
Arvanil	5.48 ± 0.13	89 ± 9	6–8	6.42 ± 0.21	135 ± 25	4	8.8**	1.5*
OLDA	4.29 ± 0.08	81 ± 14	5	5.03 ± 0.03	72 ± 3	3	5.5**	0.9
NADA	5.29 ± 0.18	30 ± 6	7	5.52 ± 0.16	68 ± 12	4	1.7	2.3**
Anandamide	4.46 ± 0.01	59 ± 1	12	4.74 ± 0.03	137 ± 5	3	1.9**	2.3**
Olvanil	4.98 ± 0.1	39 ± 7	8	5.00 ± 0.28	64 ± 24	4	1.1	1.6&*
9-HODE	>4.5	37 ± 13 @ 33μM	4	>4.5	51 ± 7 @ 33μM	3	N/A	N/A
HPPB	>5	14 ± 1 @ 10μM	2	~5.44	~115	2	N/A	N/A

**$P < 0.01$, *$P < 0.05$.

An additional set of TRPV1 antagonists, including ABT-102, AMG8562 and AMG8563, (Figure 6A–C) partially inhibited PDBu evoked CGRP release or failed to antagonize PDBu activation of TRPV1 (Figure 6D–E; capsazepine and SB366791), despite fully inhibiting capsaicin-evoked release (Figure 6; Table 3).

Finally, one TRPV1 antagonist, *N*-(3-hydroxyphenyl0-4-phenyl-benzamide (HPPB), which blocked capsaicin induced CGRP release (pIC_{50} = 5.76 ± 0.08 M), actually increased 1 μM PDBu-evoked release (pEC_{50} = 6.34 ± 0.17 M) to 162% (Figure 6; Table 3). This compound was subsequently tested as an agonist of TRPV1 in the absence of PDBu (pEC_{50} > 5 M, 14 ± 0.8% stimulation at 10 μM) or in the presence of sub-maximal PKC stimulation (pEC_{50} ~5.44 M, ~115% stimulation at 10 μM; Figure 7).

Discussion

In the current study, we have utilized a relatively high-throughput *in vitro* CGRP release assay to pharmacologically characterize native rat TRPV1 receptor function. The assay is statistically robust, straightforward to perform and enables the construction of full concentration–response curves for both TRPV1 agonist and antagonists. We show that functional TRPV1 receptors are present in rat and mouse spinal cord homogenate and are directly activated by a number of endogenous and synthetic TRPV1 ligands. In addition, spinal cord TRPV1 receptors are activated and/or sensitized by conditions promoting phosphorylation by PKA and PKC. Phosphorylation by PKC increased the potency of known TRPV1 chemical agonists in an agonist-specific manner, in addition

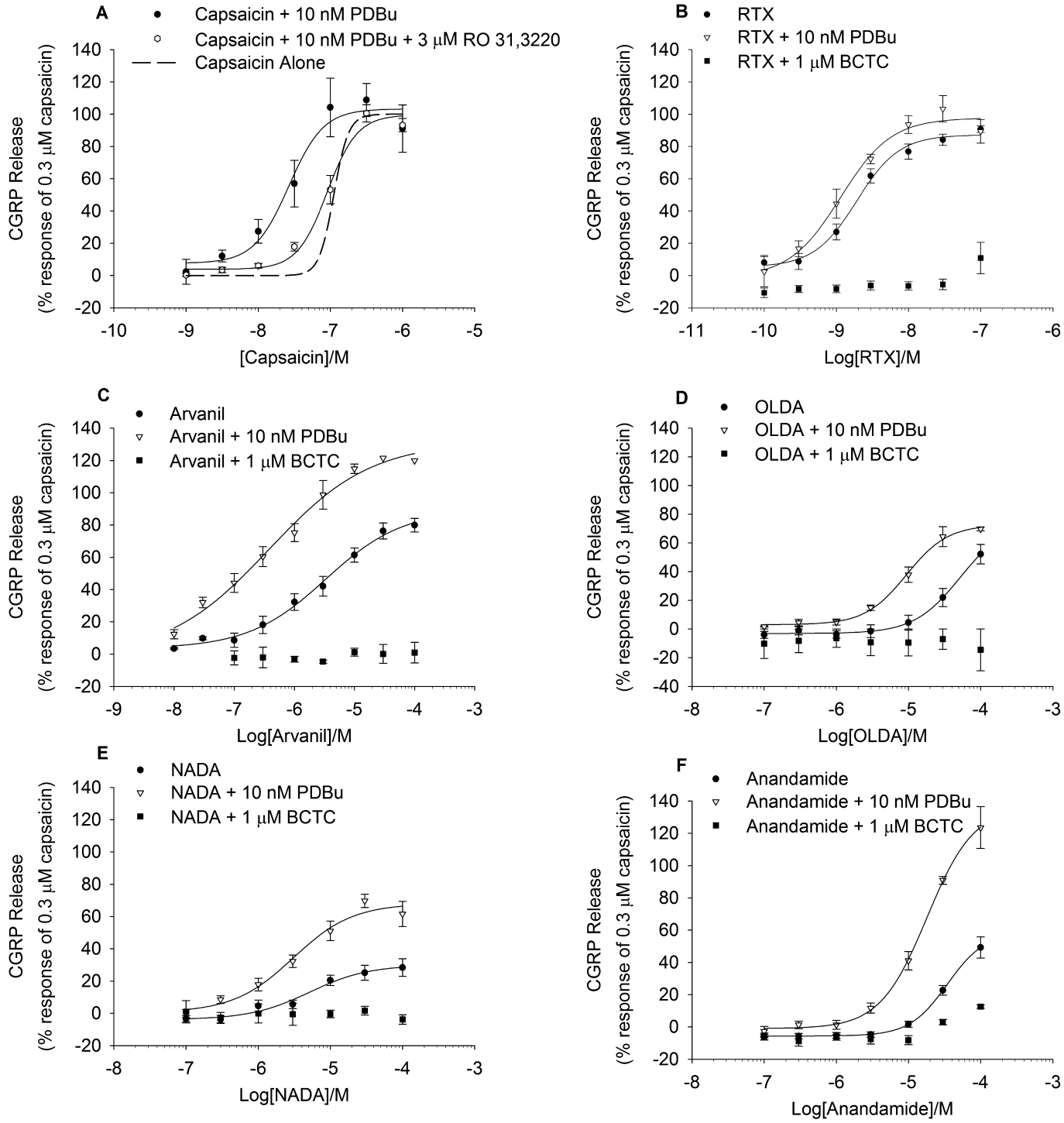

Figure 3

TRPV1 agonists evoke CGRP release with higher potency and efficacy after pre-incubation with PDBu. (A–H) Effect of PDBu pre-treatment (10 nM for 10 min) on agonist-evoked CGRP release from rat spinal cord homogenate. All agonists were incubated for 10 min. Pre-treatment with PDBu caused a leftward shift in the curve for most agonists, with degree of shift being agonist dependent and shown in Table 1. Agonist effects on tissue alone shown for reference. Responses to agonists in the presence of 1 μM of the TRPV1 antagonist BCTC are also shown. Each data point represents the mean release ± SEM from at least three separate experiments.

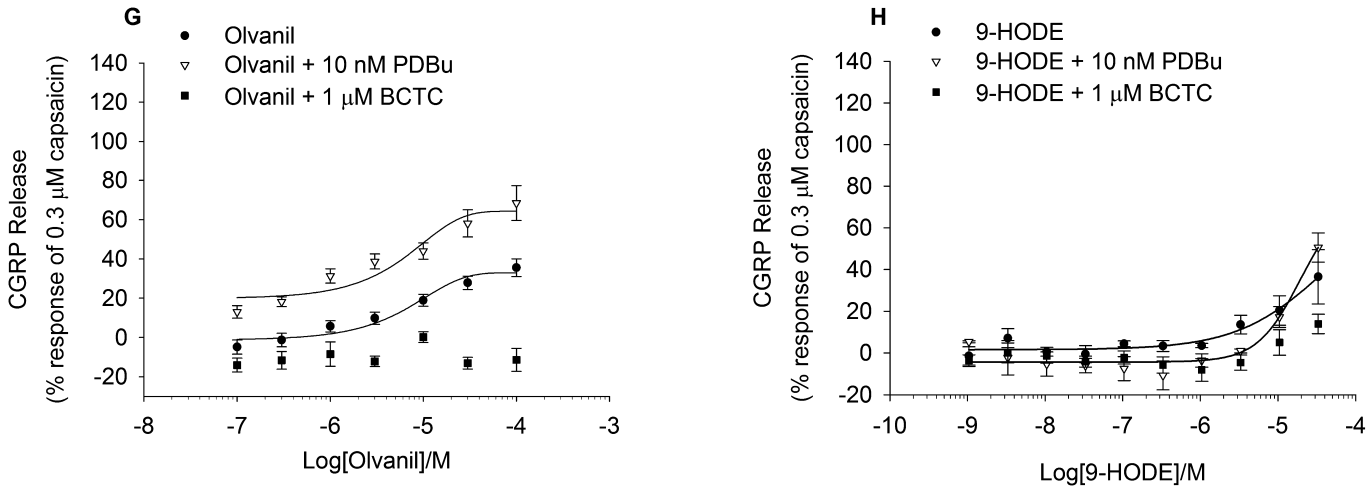

Figure 3

Continued

Table 2

Summary of the effects of TRPV1 antagonists on CGRP release elicited by maximally effective concentrations of capsaicin or PDBu

	Inhibition of 300nM capsaicin response			Inhibition of 1 μM PDBu response			
Compound	**pIC_{50} ± SEM (M)**	**Inhibition (%)**	***n***	**pIC_{50} ± SEM (M)**	**Inhibition (%)**	***n***	**Reference**
BCTC	8.46 ± 0.09	102 ± 6	3	8.93 ± 0.05**	98 ± 4	3	(Valenzano *et al.*, 2003)
AMG-517	7.33 ± 0.02	106 ± 1	3	7.92 ± 0.05**	94 ± 2	3–4	(Gavva *et al.*, 2007)
Neurogen	8.02 ± 0.07	100 ± 4	2	8.52 ± 0.1**4	101 ± 14	3–6	(Zheng *et al.*, 2006)
A425619	7.12 + 0.08	92 ± 4	3	6.63 ± 0.08*	90 ± 4	3–7	(El-Kouhen *et al.*, 2005)
A784168	6.98 ± 0.04	104 ± 3	3	6.80 ± 0.08**	95 ± 3	3–7	(Cui *et al.*, 2006)
AMG 49a	6.78 ± 0.08	102 ± 4	3	6.55 ± 0.08*	97 ± 3	3–6	(Doherty *et al.*, 2005)
SB705498	5.97 + 0.12	102 ± 9	3	5.46 ± 0.16	116 ± 12	3–6	(Gunthorpe *et al.*, 2007)
JNJ17203212	6.59 ± 0.09	98 ± 6	3	6.75 ± 0.08	98 ± 18	3	(Swanson *et al.*, 2005)
AMG-9810	5.89 ± 0.09	88 ± 5	3	6.81 ± 0.04*	84 ± 1	3	(Gavva *et al.*, 2005)

**$P < 0.01$, *$P < 0.05$.

Table 3

Summary of the effects of TRPV1 antagonists displaying differential effects on CGRP release elicited by maximally effective concentrations of capsaicin or PDBu

	Inhibition of 300 nM capsaicin response			Inhibition of 1 μM PDBu response			
Compound	**pIC_{50} ± SEM**	**Inhibition (%)**	***n***	**pIC_{50} ± SEM**	**Inhibition (%)**	***n***	**Reference**
ABT-102	7.62 ± 0.05	104 ± 4	3	8.18 ± 0.1**	60 ± 2**	2	(Surowy *et al.*, 2008)
AMG-8562	8.45 ± 0.08	100 ± 4	3	9.00 ± 0.18	19 ± 15**	3	(Lehto *et al.*, 2008)
AMG-8563	8.27 ± 0.05	104 ± 3	3	8.90 ± 0.08**	67 ± 2**	3	(Lehto *et al.*, 2008)
Capsazepine	6.15 ± 0.06	108 ± 5	3–6	>4	0	3–6	(Bevan *et al.*, 1992)
SB366791	~5	~50% @ 10μM	4	>4	0	3	(Gunthorpe *et al.*, 2004)
HPPB	5.76 ± 0.08	86 ± 9	3	6.34 ± 0.17 (EC_{50})	162 ± 9 (Efficacy)	2–3	See methods

**$P < 0.01$, *$P < 0.05$.

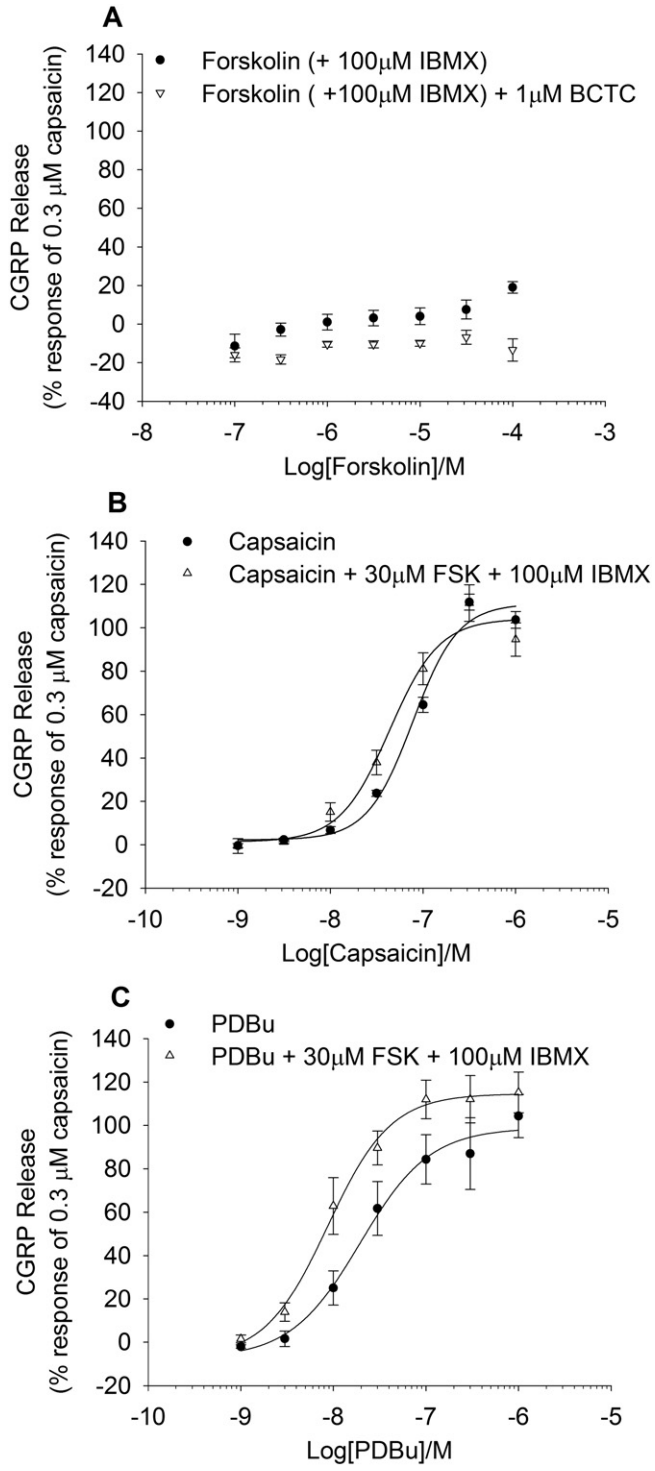

Figure 4

Effects of PKA activation on CGRP release. (A) Effects of the PKA activator forskolin in the presence of the PDE inhibitor IBMX in the spinal cord homogenate. IBMX alone did not evoke CGRP release (data not shown). Forskolin evoked CGRP release was inhibited by BCTC (1 μM). (B) Responses to capsaicin in the absence and in the presence of 30 μM forskolin + IBMX (100 μM). In the presence of forskolin + IBMX there is a 1.8-fold shift in the curve to capsaicin. 30 μM FSK and IBMX evoked a basal CGRP release of 12.7 ± 2.5%. Each data point represents the mean release ± SEM from at least three separate experiments. (C) Forskolin potentiation of PDBu responses – PDBu tested alone and in the presence of 30 μM forskolin and 100 μM IBMX. Data are displayed as a percentage of 0.3 μM capsaicin control minus relevant basal. Each data point represents the mean release ± SEM from at least three separate experiments.

to significantly altering the inhibitory profile of structurally diverse TRPV1 antagonists. A comparison of such a wide range of TRPV1 ligands in a preparation containing native receptor–effector mechanisms coupled to release in the absence, or presence of phosphorylation has not previously been performed.

The prototypic TRPV1 agonist, capsaicin, evoked CGRP release in a Ca^{2+}-dependent manner with a similar pEC_{50} to that found in CGRP release studies from spinal cord slices (Wardle *et al.*, 1997). Responses elicited by capsaicin were fully inhibited by a TRPV1 antagonist, BCTC, and were absent in tissue from mice lacking the TRPV1 receptor, confirming that capsaicin-induced CGRP release was indeed mediated by the TRPV1 receptor. The 96-well format of the assay made it possible to directly compare the potencies and efficacies of a number of other TRPV1 agonists including olvanil, anandamide, OLDA, arvanil, NADA, 9-HODE and RTX. Application of a sub-maximal concentration of PDBu resulted in increases in potency and efficacy of these agonists at TRPV1. The degree of shift in pEC_{50} was agonist dependent, with arvanil showing the largest shift and RTX, NADA and 9-HODE shifting the least. RTX is structurally related to phorbol esters and has been shown to directly activate PKC in dorsal root ganglion neurones (Harvey *et al.*, 1995); however, we do not believe that this is the case here, as pre-incubation with a PKC inhibitor, Ro 31-8220, did not affect RTX induced CGRP release (data not shown). In line with our findings, alteration of TRPV1 agonist pharmacology by phosphorylation has been reported previously in studies utilizing recombinant TRPV1. A PKC activator, phorbol myristate acid (PMA), was shown to increase the efficacy of two partial agonists, JYL827 and JYL1511 (Wang *et al.*, 2003). Whilst a protein phosphatase inhibitor, cyclosporine A, employed to inhibit TRPV1 de-phosphorylation, led to increased potencies (1.1- to 7.8-fold) of a panel of TRPV1 agonists and increased efficacy of JYL1511 (Pearce *et al.*, 2008).

The PKC activator PDBu, applied alone, evoked a concentration-dependent increase in CGRP release of comparable magnitude with capsaicin and was inhibited by BCTC or a PKC inhibitor, Ro 31-8220. PDBu did not elicit release above basal levels in experiments performed using tissue from mice deficient in the TRPV1 receptor. In addition, the inactive analogue 4α-PDD did not elicit significant release. PKC activation may act to sensitize CGRP synaptic release in a number of ways, including direct phosphorylation of the TRPV1 channel. As the findings in the present study replicate in a simpler recombinant system and are consistent with the findings of Premkumar *et al.* (2004), who reported that the sensitizing/activating effects of PDBu on recombinant TRPV1 were significantly reduced or lost in a TRPV1 double PKC phosphorylation site mutant, we hypothesize that direct phosphorylation of TRPV1 likely underlies the effects observed in the native system as well. Of note in the present

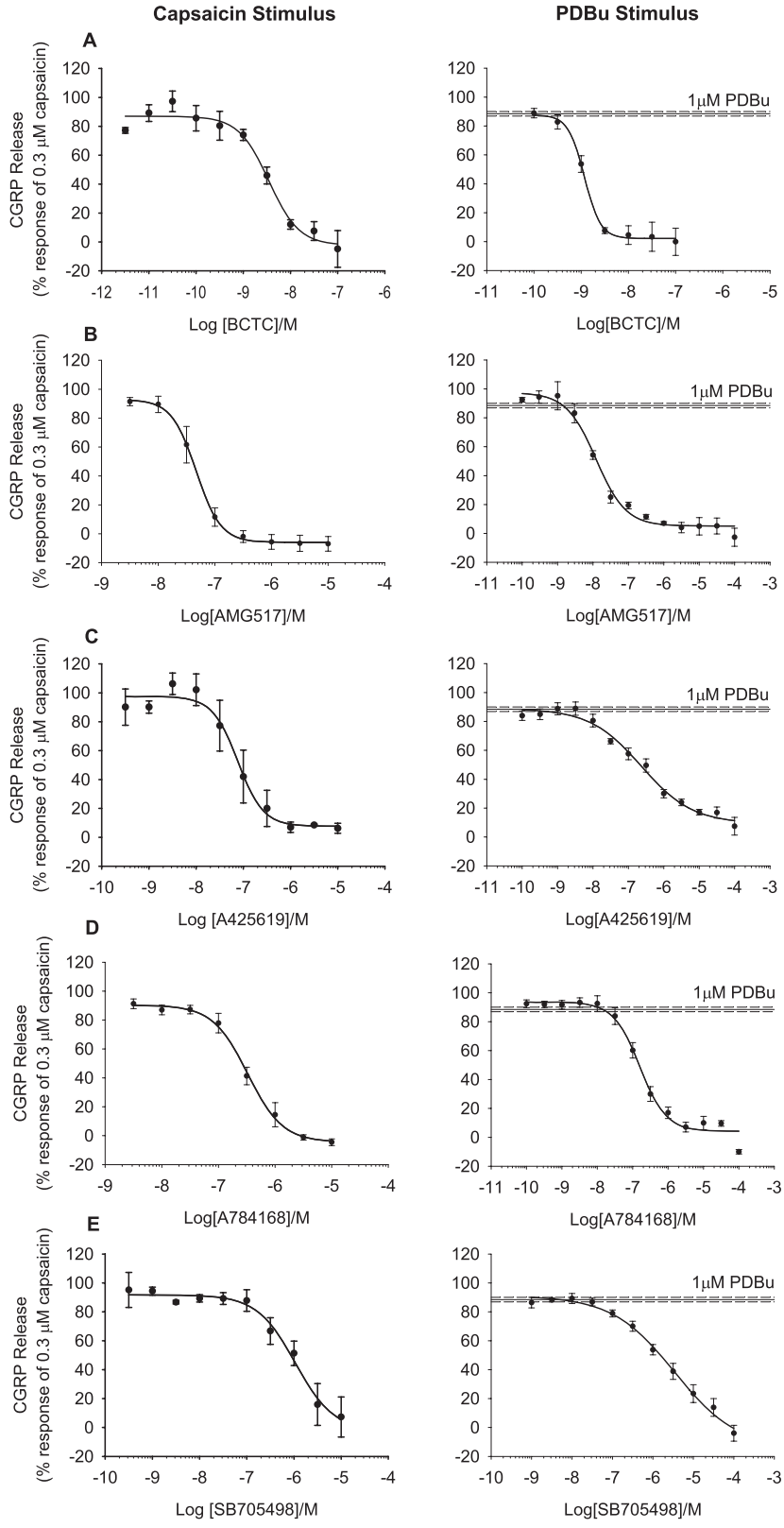

Figure 5

TRPV1 antagonists eliciting full inhibition of capsaicin or PDBu-evoked CGRP release. (A–E) Antagonists were pre-incubated for 10 min and subsequently applied together with 0.3 μM Capsaicin or 1 μM PDBu. Data are displayed as a percentage of 0.3 μM capsaicin control minus relevant basal with the response to 1 μM PDBu shown for comparison. Each data point represents the mean release ± SEM from at least three separate experiments.

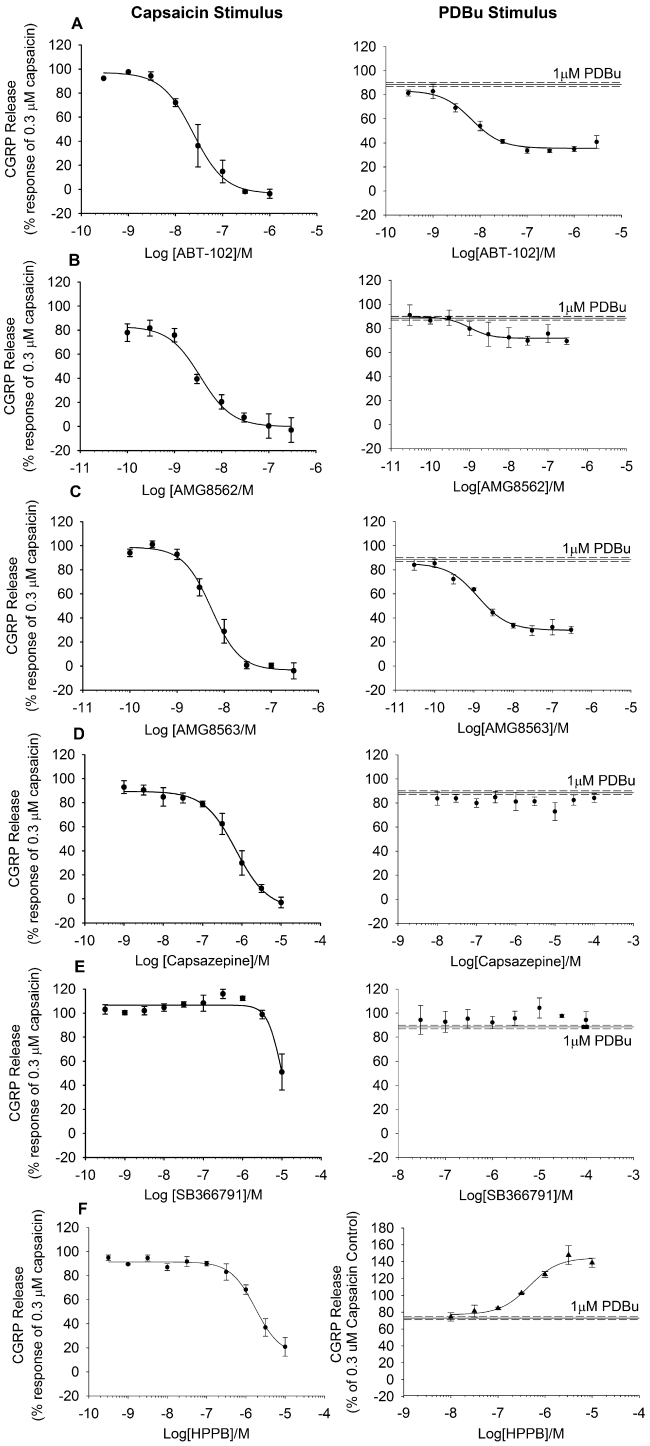

Figure 6

TRPV1 antagonists showing partial inhibition of the response to PDBu. Antagonists were pre-incubated for 10 min and subsequently applied together with 0.3 μM (A) Capsaicin or 1 μM PDBu (B). Data are displayed as a percentage of 0.3 μM capsaicin control minus relevant basal. Each data point represents the mean release ± SEM from at least three separate experiments.

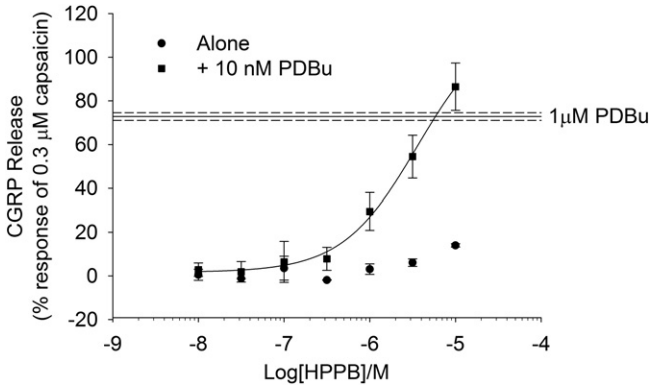

Figure 7

TRPV1 antagonist exhibiting an agonistic-type profile on CGRP release. Antagonist (HPPB) was applied alone or after 10 min pre-incubation with 10 nM PDBu. Data are displayed as a percentage of 0.3 μM capsaicin control minus relevant basal. Each data point represents the mean release ± SEM from two separate experiments.

study, the maximal PDBu-evoked response is lower in the recombinant (65 ± 3%) versus the native (94 ± 8 or 89 ± 5%) preparation, relative to a maximal concentration of capsaicin, perhaps suggesting a contribution from additional elements in the latter preparation.

Whilst the effects of a PKA activator, forskolin, were not as pronounced as the effects of PDBu, at high concentrations, forskolin induced BCTC-sensitive CGRP release. Moreover, when PDBu was assayed in the presence of forskolin and IBMX, there was a twofold increase in potency, suggesting a synergistic relationship between PKA and PKC. Taken together, these results imply that in CGRP-releasing terminals in the spinal cord, there is a tight relationship between PKC/PKA and TRPV1; and that phosphorylation of the receptor is sufficient to induce activation at temperatures at or below body temperature, as shown previously (Sikand and Premkumar, 2007). In line with this, aberrant TRPV1 activity has been shown to underlie the increased basal neurotransmitter release observed in spinal cord from pain state animals (Tohda *et al.*, 2001; Kanai *et al.*, 2005; Schicho *et al.*, 2005; Puttfarcken *et al.*, 2010).

By comparing the profile of a panel of literature TRPV1 antagonists to inhibit capsaicin or PDBu-induced responses, distinct categories of TRPV1 antagonist were revealed. Pharmacological characterizations of TRPV1 antagonists routinely employ chemical agonists (e.g. capsaicin), pH and heat to activate TRPV1. Utilizing these activators has led to the identification of diverse TRPV1 antagonist profiles, from TRPV1 antagonists that block all three modes of activation to antagonists that block capsaicin, but vary in their profiles against pH or heat responses. In the current study, antagonists previously reported to inhibit TRPV1 activation by capsaicin, pH and heat (Gavva *et al.*, 2005; 2007; Gunthorpe *et al.*, 2007; Broad *et al.*, 2008) generally also fully inhibited phosphorylated TRPV1, with IC_{50} values comparable with those for inhibition of capsaicin responses (Figure 5). SB366791 appears to be an exception as it was reported to block capsaicin, heat and pH activated TRPV1 (Gunthorpe *et al.*, 2004) but failed to inhibit phosphorylated TRPV1 in the current study, despite inhibition

of capsaicin mediated responses. However, in our hands, SB366791 failed to block pH activated TRPV1 and only partially inhibited heat activated TRPV1 (Broad *et al.*, 2008). Likewise, whilst capsazepine failed to inhibit rat TRPV1 in some reports (Bevan *et al.*, 1992; Vyklicky *et al.*, 1998), it fully blocked all three activation modes at rat TRPV1 in others (Broad *et al.*, 2008). In the present study, capsazepine failed to reduce phosphorylated TRPV1. Another anomaly was seen with A425619, which gave comparable potency and extent of block of capsaicin and phosphorylation activated TRPV1, but is reported as a more potent and/or efficacious blocker of capsaicin versus pH (Broad *et al.*, 2008; Wong and Gavva, 2009), but see also El-Kouhen *et al.*, 2005. Finally, AMG 8562, AMG 8563 and ABT-102, partially inhibited PDBu-evoked CGRP release, despite full inhibition of capsaicin responses. In line with this, previous data indicate that these ligands all block capsaicin-induced responses in recombinant systems; however, their profile versus heat and pH varies from full block through to potentiation (Lehto *et al.*, 2008; Surowy *et al.*, 2008). The incomplete overlap of antagonist pharmacology for phosphorylation activated TRPV1 compared with other activation modes suggests phosphorylation may promote a distinct state of activation, although further work would be required to confirm this.

The most striking finding was the contrasting profile of HPPB, which inhibited capsaicin-evoked CGRP release, but potentiated phosphorylation-evoked CGRP release. When assessed as an agonist of TRPV1 in its naïve state, there was little discernable agonism, but in the presence of sub-maximal PKC stimulation, the efficacy of release was increased 8-fold. In contrast, other full and partial agonists studied showed only a 0.9- to 2.3-fold increase in efficacy, respectively, in the presence of sub-maximal PKC stimulation. We have previously reported a similar finding with the CB2 agonist GW405833 under naive and phosphorylating conditions (Schuelert *et al.*, 2010). Underlying mechanisms may include an increase in the sensitivity and maximal response of the receptor, activation of silent channels or recruitment of new channels to the plasma membrane as well as changes in channel desensitization (Premkumar *et al.*, 2004; Sculptoreanu *et al.*, 2008; Studer and McNaughton, 2010).

In summary, the results presented herein demonstrate that this assay is a valuable tool in the analysis of both agonist and antagonist responses at native TRPV1 under naive or phosphorylating conditions, employed to simulate a pain state. The data suggest that in addition to capsaicin, heat and pH, a distinct mode of TRPV1 activation, phosphorylation, may exist. Profiling of phosphorylated TRPV1 may be important to include when designing TRPV1 ligands, as it may provide further insights into differential *in vivo* efficacy and adverse profiles. Additionally, by identification of ligands with agonist properties differentially modulated by, or apparent only, in a context-dependent manner, the possibility of selectively targeting pathological state TRPV1 moves closer.

Acknowledgements

We thank Roger Moore for statistical analysis of CGRP release data and Dr Emanuele Sher for helpful discussions and comment.

Conflict of interest

The authors declare no conflicts of interest. Eli Lilly does not sell any of the drugs or devices mentioned in this article.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 (A) Comparison of PDBu-evoked Ca^{2+} influx responses at 21°C or 37°C in CHO cells transfected with the rTRPV1 receptor, recorded at either 21°C (•) or 37°C (r) (B) Inhibition of responses to 1μM PDBu with BCTC.

Data are displayed as a percentage of the 10μM capsaicin control response minus the response to buffer alone. Each data point represents the mean response ± SEM from 2–5 separate experiments.

Figure S2 TRPV1 antagonists eliciting full inhibition of capsaicin or PDBu-evoked CGRP release. Antagonists were pre-incubated for 10 minutes and subsequently applied together with 0.3 μM Capsaicin or 1 μM PDBu. Data are displayed as a percentage of 0.3 μM capsaicin control minus relevant basal with the response to 1 μM PDBu shown for comparison. Each data point represents the mean release ± SEM from at least 3 separate experiments.

Figure S3 Correlation plot comparing potency of antagonists eliciting full inhibition of capsaicin and PDBu-evoked CGRP release.