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RESEARCH PAPER

The tyrosine kinase inhibitor bafetinib inhibits PAR2-induced activation of TRPV4 channels *in vitro* and pain *in vivo*

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BACKGROUND AND PURPOSE

Protease-activated receptor 2 (PAR2) is expressed on nociceptive neurons, and can sensitize transient receptor potential (TRP) ion channels to amplify neurogenic inflammation and pain. The mechanisms by which this occurs are not fully understood. PAR2 causes receptor-operated activation of TRPV4 channels and TRPV4 null mice have attenuated PAR2-stimulated neurogenic inflammation and mechanical hyperalgesia. Here we investigate the intracellular signalling mechanisms underlying PAR2-induced TRPV4 channel activation and pain.

EXPERIMENTAL APPROACH

Responses of non-transfected and TRPV4-transfected HEK293 cells to agonists of PAR2 (trypsin and SLIGRL) and TRPV4 channels (GSK1016790A) were determined using calcium imaging. Inhibitors of TRPV4 channels (HC067047), sarcoendoplasmic reticulum calcium transport ATPase (thapsigargin), $G\alpha_q$ (UBO-QIC), tyrosine kinases (bafetinib and dasatinib) or PI3 kinases (wortmannin and LY294002) were used to investigate signalling mechanisms. *In vivo* effects of tyrosine kinase inhibitors on PAR_2-induced mechanical hyperalgesia were assessed in mice.

KEY RESULTS

In non-transfected HEK293 cells, PAR2 activation transiently increased intracellular calcium ($[Ca^{2+}]_i$). Functional expression of TRPV4 channels caused a sustained increase of $[Ca^{2+}]_i$, inhibited by HC067047, bafetinib and wortmannin; but not by thapsigargin, UBO-QIC, dasatinib or LY294002. Bafetinib but not dasatinib inhibited PAR2-induced mechanical hyperalgesia *in vivo*.

CONCLUSIONS AND IMPLICATIONS

This study supports a role for tyrosine kinases in PAR2-mediated receptor-operated gating of TRPV4 channels, independent of $G\alpha_q$ stimulation. The ability of a tyrosine kinase inhibitor to diminish PAR2-induced activation of TRPV4 channels and consequent mechanical hyperalgesia identifies bafetinib (which is in development in oncology) as a potential novel analgesic therapy.

Abbreviations

4αPDD, 4α-phorbol 12,13-didecanoate; $[Ca^{2+}]_i$, intracellular calcium concentration; DMSO, dimethyl sulfoxide; GSK, GSK1016790A; HEK, human embryonic kidney 293 cell; IP_3, inositol trisphosphate; NT, non-transfected; PAR, protease-activated receptor; SERCA, sarcoendoplasmic reticulum calcium transport ATPase; TRP, transient receptor potential; TRPA1, transient receptor potential ankyrin 1; TRPV1, transient receptor potential vanilloid 1; TRPV4, transient receptor potential vanilloid 4; WNK, lysine-deficient kinase

Introduction

The family of transient receptor potential (TRP) ion channels has many important physiological roles in a range of tissues and cell types. There are several reports of the capacity of exogenous environmental and synthetic stimuli to directly bind and gate TRP channels (Caterina *et al.*, 1997; Peier *et al.*, 2002; Watanabe *et al.*, 2002a; Bautista *et al.*, 2006; Vogt-Eisele *et al.*, 2007; Thorneloe *et al.*, 2008; channel nomenclature follows Alexander *et al.*, 2013a). These stimuli are relevant in tissues such as the skin, lung and gut that come in direct contact with exogenous factors, but are not likely to be otherwise physiologically relevant. Few endogenous agonists have been identified that directly regulate TRP channels, and for most TRP ion channels, the physiological mechanisms of channel gating are unknown.

The first *trp* channel was discovered in *Drosophila* (Montell and Rubin, 1989; Wong *et al.*, 1989), and characterized as a receptor-operated channel that was activated downstream of the rhodopsin light receptor, resulting in sustained activation or 'receptor potential' (Minke and Selinger, 1996). It is therefore possible that the physiological mechanism of mammalian TRP channel gating is also via signalling pathways initiated by activation of membrane-bound receptors. Indeed, GPCRs that induce neurogenic inflammation and pain can indirectly activate and 'sensitize' TRP channels via intracellular signalling pathways. For example, agonists of the protease-activated receptor PAR2 augment subsequent responses to agonists of TRPA1, TRPV1 and TRPV4 channels in model cells and nociceptive neurons, and thereby enhance the release of neuropeptides that stimulate neurogenic inflammation and pain (Amadesi *et al.*, 2004; Dai *et al.*, 2004; 2007; Grant *et al.*, 2007). Moreover, pretreatment with PAR2 agonists or an 'inflammatory soup' has been demonstrated to potentiate subsequent pain behaviour stimulated by activation of TRPA1, TRPV1 and TRPV4 channels (Amadesi *et al.*, 2004; Alessandri-Haber *et al.*, 2006; Dai *et al.*, 2007).

The PARs are a family of four GPCRs that regulate many pathophysiological processes including haemostasis, inflammation, pain and healing (Ossovskaya and Bunnett, 2004; Ramachandran *et al.*, 2012; Poole *et al.*, 2013; receptor nomenclature follows Alexander *et al.*, 2013b). Tissue injury and inflammation induce the activation of proteases from the circulation, epithelial cells and immune cells. Activated proteases regulate cells by cleaving PARs at a specific site on their extracellular N-terminal domains. Cleavage reveals a tethered ligand that binds to and activates the receptor (Ossovskaya and Bunnett, 2004; Poole *et al.*, 2013). PAR2 is activated by pro-inflammatory proteases such as trypsin, tryptase and kallikreins, and proteases released from granulocyte neutrophils, bacteria and insects. The PAR2 receptor can couple to the G-proteins $G\alpha_{i/o}$, $G\alpha_s$ and $G\alpha_{q/11}$ to activate intracellular signalling cascades (Ramachandran *et al.*, 2009). It is expressed in primary afferent neurons in the dorsal root ganglia, and stimulates the release of neuropeptides that induce mechanical and thermal hyperalgesia (Steinhoff *et al.*, 2000; Vergnolle *et al.*, 2001). We have previously shown that PAR2-induced thermal hyperalgesia is attributable to the sensitization of TRPV1 (Amadesi *et al.*, 2004), while mechanical hyperalgesia is linked to TRPV4 channels (Grant *et al.*, 2007; Sipe *et al.*, 2008).

The TRPV4 channel is a polymodal non-selective cation channel that is involved in the sensation of pain, mechanical stress, osmotic stress and temperatures above 27°C (Liedtke *et al.*, 2000; Strotmann *et al.*, 2000; Güler *et al.*, 2002; Watanabe *et al.*, 2002b; Liedtke and Friedman, 2003; Suzuki *et al.*, 2003; Grant *et al.*, 2007). TRPV4 channels are activated by endogenous fatty acids, arachidonic acid and its metabolites, and was recently shown to mediate pain induced by UVB exposure in mice (Watanabe *et al.*, 2003; Moore *et al.*, 2013; Poole *et al.*, 2013). Synthetic agonists and inhibitors (e.g. HC067047 and RN1734) (GSK1016790A and 4α-PDD) (Vriens *et al.*, 2007; Thorneloe *et al.*, 2008; Poole *et al.*, 2013) (Vincent *et al.*, 2009; Everaerts *et al.*, 2010) for TRPV4 channels have also been described. However, the physiological modes of TRPV4 channel activation are not well understood. This is an important area of investigation because TRPV4 channels are widely expressed and contribute to diverse pathophysiological processes (Wegierski *et al.*, 2009). Although PAR2 activation and subsequent signalling have been shown to sensitize TRPV4 channels (Alessandri-Haber *et al.*, 2006; Grant *et al.*, 2007), few studies have examined GPCR-dependent activation of TRPV4 channels. Our group recently demonstrated that stimulation of PAR2 could activate TRPV4 channels, leading to a sustained increase of intracellular calcium in HEK293 cells expressing TRPV4 channels that was not observed in non-transfected HEK293 cells (Poole *et al.*, 2013). Thus, PAR2 activation can lead to receptor-operated gating of TRPV4 channels, which we refer to as 'coupling'. TRPV4 also mediates PAR2-induced responses in mouse dorsal root ganglion (DRG) neurons (Poole *et al.*, 2013). PAR2-induced phosphorylation of TRPV4 is a potential mechanism of coupling. Several sites that can be phosphorylated and affect function have been identified on the intracellular N- and C-termini of TRPV4 channels, some of which are near regulatory regions such as Y110 near the N-terminal proline-rich domain (Xu *et al.*, 2003; Wegierski *et al.*, 2009; Lee *et al.*, 2010; Peng *et al.*, 2010). The significance of TRPV4 phosphorylation in the N-terminal of TRPV4 channels is not understood; however, the importance of this region is illustrated by the finding that mutations in the ankyrin repeat region of the N-terminus result in a range of pathologies, which include arthropathy, skeletal muscle dysplasia and axonal neuropathies (Fecto *et al.*, 2011; Lamandé *et al.*, 2011). This suggests that TRPV4 channel phosphorylation at these sites could produce different outcomes in different tissues, depending on what interacting proteins are present. In particular, Y110 in the N-terminus and Y805 in the C-terminus have been identified as key for sensitizing TRPV4 channels to hypo-osmolarity, mechanical stress and temperature (Wegierski *et al.*, 2009). We found that Y110 is important for PAR2 coupling to TRPV4 channels, implicating tyrosine phosphorylation as a signalling mechanism in this phenomenon (Poole *et al.*, 2013). We also demonstrated small inhibitory effects of blocking selected intracellular signalling pathways (PLA_2 and cytochrome P450 epoxygenase) on PAR2-TRPV4 channel coupling (Poole *et al.*, 2013). However, these data are difficult to interpret due to the high concentrations of inhibitor required to block coupling, an observed reduction in PAR2-mediated intracellular calcium release, and inhibition of direct activation of TRPV4 channels by a selective agonist (Poole *et al.*, 2013). By contrast, the Src family kinase inhibi-

tor Src inhibitor 1 leads to a small but significant inhibition of PAR2-TRPV4 coupling, without affecting the transient phase of PAR2 intracellular calcium release. These data support our assertion that tyrosine kinases are important in PAR2-TRPV4 coupling and, in part, implicate a role for Src kinases. However, other pathways are known to be activated downstream of PAR2 receptor stimulation, which could also contribute to TRPV4 activation. PAR2-mediated sensitization of TRPV4 channels has been suggested to involve coupling of the receptor to $G\alpha_{q/11}$, activation of PLC_{β} and the release of inositol trisphosphate (IP_3), which stimulates the release of intracellular calcium stores from the endoplasmic reticulum (Grant *et al.*, 2007). Alternately, PAR2 has been shown to induce TRPV4-mediated calcium influx via $G\alpha_{i/o}$ signalling in the airways, involving $PLC\beta_3$ and PI3K (Li *et al.*, 2011). PI3Ks are also known to be activated by the $G\alpha_q$ pathway, and generate second messengers that lead to the activation of lysine-deficient kinases (WNKs). WNKs have been shown to modulate TRPV4 channel activity (Fu *et al.*, 2006), and may therefore also play a role in PAR2-induced activation of TRPV4 channels.

Here, we show that PAR2-TRPV4 coupling does not depend on release of intracellular calcium, activation of $G\alpha_q$-mediated signalling pathways or PI3Ks. Further, we identify and characterize a tyrosine kinase inhibitor, currently in clinical trials as a cancer treatment, that is also efficacious in inhibiting PAR2-TRPV4 coupling *in vitro*, and attenuates PAR2-induced mechanical hyperalgesia *in vivo*. These findings implicate tyrosine kinases and subsequent phosphorylation of TRPV4 channels as the mechanism by which PAR2 couples to open TRPV4, and could lead to identification of novel therapeutic targets for pain.

Methods

Cell lines

HEK293 T-Rex cell lines stably expressing human TRPV4 channels were generated using a tetracycline-inducible system as described in Poole *et al.* (2013). Briefly, Flp-In™ T-Rex™ HEK293 cells (Life Technologies, Mulgrave, Australia) were transfected with pcDNA5/FRT/TO containing human TRPV4 (TRPV4 HEK) using Lipofectamine 2000 (Life Technologies). Cells were grown in DMEM (Life Technologies) containing 10% tetracycline-free FBS, blasticidin (5 $\mu g \cdot mL^{-1}$) and hygromycin (100 $\mu g \cdot mL^{-1}$). Alternatively, non-transfected control HEK293 cells (NT HEK) were grown in DMEM containing 10% tetracycline-free FBS and blasticidin (5 $\mu g \cdot mL^{-1}$). A freeze-thaw assay was used where cells were frozen (–150°C) at 1.6×10^7 cells/mL in freezing medium (70% DMEM, 20% FBS and 10% DMSO), thawed and plated prior to use.

Intracellular calcium assay

TRPV4 and NT HEK cells were thawed, re-suspended in DMEM with 10% FBS at 4×10^5 cells/mL, and seeded onto poly-L-lysine (100 $\mu g \cdot mL^{-1}$) coated 96-well plates (60 000 cells/well) and cultured for 48 h. At 32 h, tetracycline (0.1 $\mu g \cdot mL^{-1}$) was added to the medium for 16 h to induce TRPV4 expression. NT HEKs were also treated with tetracycline to replicate culture conditions. On the day of experiment, cells were washed with HBSS containing: 140 mM NaCl, 5 mM KCl, 10 mM HEPES, 11 mM D-glucose, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 2 mM probenecid; pH 7.4 at 37°C prior to loading with Fura2-AM ester (2.5 μM) and pluronic acid (0.5 μM) in HBSS for 45–60 min at 37°C and 5% CO_2 in the dark. After loading, cells were washed twice with HBSS.

Fluorescence was measured at 340 and 380 nm excitation and 510 nm emission wavelengths using a FlexStation 3 plate reader (Molecular Devices, Sunnyvale, CA, USA) as described (Veldhuis *et al.*, 2012). Results are expressed as the 340/380 nm fluorescence ratio, which is proportional to intracellular calcium ($[Ca^{2+}]_i$) levels. Fluorescence was recorded for 15 s prior to addition of a drug to establish a baseline reading. The first agonist was added at 15 s (GSK1016790A 1 nM–1 μM; PAR2-activating peptide SLIGRL 100 nM–100 μM; or trypsin 0.1–100 $U \cdot mL^{-1}$), followed in some experiments by the addition of a submaximal concentration of the TRPV4-selective agonist GSK1016790A (30 nM) at 80 s. For inhibitor studies, cells were incubated for 30 min with a TRPV4 antagonist (HC067047 0.01–1 μM), sarcoendoplasmic reticulum calcium transport ATPase (SERCA) inhibitor (thapsigargin 1 μM), selective $G\alpha_q$ inhibitor (UBO-QIC 100 nM), tyrosine kinase inhibitors (bafetinib 1–10 μM, dasatinib 1–10 μM), PI3K inhibitors (wortmannin 0.1–10 μM, LY294002 10–50 μM), a selective MEK1/2 inhibitor (U0126 1–10 μM) or vehicle (control) before assay. Submaximal concentrations of agonists that gave reliable and robust responses were chosen from the concentration–response curves to investigate the coupling response. Appropriate concentrations of antagonist were chosen from concentration–response curves, or from the available literature, to test against other agonists.

Murine inflammatory pain model

Animals. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Monash University. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 34 animals were used in the experiments described here.

C57BL/6 mice were obtained from Monash Animal Research Platform, Monash University. Mice (male, 6–10 weeks) were maintained in a temperature-controlled environment with a 12 h light/dark cycle and free access to food and water.

Pain behaviour. Mice were placed in individual cylinders on a mesh stand. Mice were acclimatized to the experimental room, restraint apparatus and investigator for 2 h periods on 3 successive days before experiments. The investigator was unaware of the test agents. To assess mechanical pain, paw withdrawal to stimulation of the plantar surface of the hind paw with graded von Frey filaments (0.078, 0.196, 0.392, 0.686, 1.569, 3.922, 5.882, 9.804, 13.725, 19.608 mN) was determined using the 'up-and-down' paradigm as described (Chaplan *et al.*, 1994; Alemi *et al.*, 2013). In this analysis, an increase in the filament stiffness that is required to induce paw withdrawal indicates mechanical analgesia, whereas a decrease in the filament stiffness required to induce

withdrawal indicates mechanical hyperalgesia. On the day of the study, von Frey scores were obtained before drug treatment to establish a baseline threshold for each animal (T = 0). Mice were then treated with bafetinib (10 mg·kg^{-1}), dasatinib (10 mg·kg^{-1}) or vehicle (1% DMSO, Cremophor EL) by gavage (100 µL). After 30 min, mice were sedated (5% isoflurane) and received intraplantar injection into the left hind paw of either the PAR2-activating peptide (SLIGRL-NH2, 1 µg) or the TRPV4 channel agonist (GSK1016790A, 65 ng). von Frey responses were recorded from the injected (left) and uninjected (right) hind paws for up to 4 h after injection. Results are expressed as % of baseline values.

Data analysis

Data were analysed using GraphPad Prism 6 software. All data are presented as mean ± SEM, with a P-value < 0.05 considered to be significant.The data from calcium imaging studies were expressed as sigmoidal curves fitted to agonist concentration responses and the treated EC_{50s} compared to vehicle control using one-way ANOVA. Due to the higher baseline fluorescence (F_{340}/F_{380} ratio) observed in TRPV4-transfected compared with NT HEKs, agonist peaks and coupling analysis were determined based on normalized data by subtracting the baseline fluorescence (average fluorescence from 0 to 15 s). Agonist peak responses were compared by one-way ANOVA with Dunn's *post hoc* test for comparisons of three or more variables, or unpaired *t*-test for comparison between two variables. Coupling of PAR2 to TRPV4 channels using normalized data at the time point which showed the greatest effect (i.e. 41 s for SLIGRL and 46 s for trypsin) was compared using one-way ANOVA with Dunn's *post hoc* test for comparisons of three or more variables, or unpaired *t*-test for comparison between two variables. Data from the i*n vivo* pain assay were analysed by comparisons of multiple groups using two-way ANOVA, followed by Dunnett's *post hoc* test.

Materials

The synthetic mouse/rat PAR2-activating peptide (SLIGRL-NH_2) that mimics the tethered ligand domain was from CPC Scientific (San Jose, CA, USA). Porcine trypsin, GSK1016790A and thapsigargin were purchased from Sigma-Aldrich (Sydney, Australia). The TRPV4 channel antagonist HC067047 was from Tocris Bioscience (Bristol, UK), and Fura2-AM from Life Technologies (Australia Pty Ltd.). Bafetinib and dasatinib were synthesized by Euroasia Trans Continental (Mumbai, India). UBO-QIC was purchased from the Institute of Pharmaceutical Biology, University of Bonn (Germany).

SLIGRL and trypsin stock solutions were made in distilled H_2O; all other stock solutions were made in DMSO and stored at –20°C until use. On the day of experiment, stocks were diluted 1/1000 in HBSS buffer, and corresponding vehicles (0.1% v/v dH_2O and 0.1% v/v DMSO) were used as a control.

Results

PAR2 agonists couple to open TRPV4 ion channels in HEK293 cells

NT and TRPV4 HEK293 cells were initially characterized using the selective TRPV4 agonist GSK1016790A and antagonist HC067047. GSK1016790A produced a concentration-dependent increase in peak $[Ca^{2+}]_i$ in TRPV4, but not NT HEKs (Supporting Information Fig. S1a), thus indicating that HEK293 cells do not endogenously express TRPV4 channels, and that the TRPV4-transfected cells were functional. The GSK1016790A response curve was concentration-dependently shifted to the right with the HC067047 inhibitor (Supporting Information Fig. S1b), without effect on the peak GSK1016790A response, indicating competitive inhibition of the TRPV4 ion channel. The GSK1016790A EC_{50} was significantly shifted from 42 ± 4.8 nM (vehicle control) to 727 ± 21 nM, with 1 µM HC067047 (P < 0.05). A submaximal concentration of 30 nM GSK1016790A was used in further experiments.

Concentration–response curves were established for the endogenous PAR2 agonist trypsin and selective synthetic PAR2 agonist SLIGRL (Supporting Information Fig. S2). The curves for both agonists were similar in both NT and TRPV4 HEKs, with no significant difference between peaks $[Ca^{2+}]_i$ at any concentration tested. However, when examining the response to the PAR2 agonists over time, it was observed that TRPV4-transfected HEKs displayed a sustained phase of high $[Ca^{2+}]_i$ in comparison to the transient nature of the PAR2 response in NT HEKs (Supporting Information Fig. S3a,c). This is consistent with our previous observations, and is a phenomenon we have termed 'coupling' (Poole *et al.*, 2013). The difference in $[Ca^{2+}]_i$ was compared at the time point which showed the greatest coupling effect (41 s for SLIGRL and 46 s for trypsin). For SLIGRL, the baseline-adjusted F_{340}/F_{380} ratio was significantly higher at 10, 30 and 100 µM (0.69 ± 0.04, 0.96 ± 0.07, and 1.43 ± 0.10, respectively) in TRPV4 HEKs compared with the NT HEK control (0.34 ± 0.05, 0.33 ± 0.05, and 0.55 ± 0.05, respectively); and for trypsin at 10, 30 and 100 U·mL^{-1} (0.87 ± 0.03, 1.30 ± 0.08, and 1.47 ± 0.10, respectively) in TRPV4 HEKs compared with the NT HEK control (0.53 ± 0.04, 0.76 ± 0.01, and 0.81 ± 0.05, respectively) (P < 0.05; Supporting Information Fig. S3b,d). Submaximal concentrations of each agonist which also showed significant coupling were chosen for further experiments (30 µM SLIGRL and 30 U·mL^{-1} trypsin).

We have previously established that the sustained $[Ca^{2+}]_i$ response was due to influx of calcium from the extracellular compartment, and that the non-selective TRP channel blocker ruthenium red also inhibited this response (Poole *et al.*, 2013). Here we demonstrate that the selective TRPV4 antagonist HC067047 concentration dependently inhibits the coupling response for SLIGRL and at 1 µM HC067047 significantly inhibits the coupling response for both SLIGRL (F_{340}/F_{380} ratio 0.33 ± 0.03) and trypsin (0.19 ± 0.02) compared with TRPV4 HEK vehicle controls (0.72 ± 0.08 and 0.68 ± 0.03 respectively) (P < 0.05). HC067047 did not affect the peak PAR2 calcium response (P > 0.05) which is due to release of intracellular calcium stores from the endoplasmic reticulum, and indicates that the HC067047 compound is not affecting the initial GPCR-mediated PAR2 response (Figure 1).

PAR2-TRPV4 coupling is not mediated by intracellular calcium release or $G\alpha_q$ signalling

$G\alpha_{q/11}$ signalling has been linked to TRPV4 channel sensitization downstream of PAR2 activation (Grant *et al.*, 2007). Stimulation of this pathway leads to PLC-mediated signalling

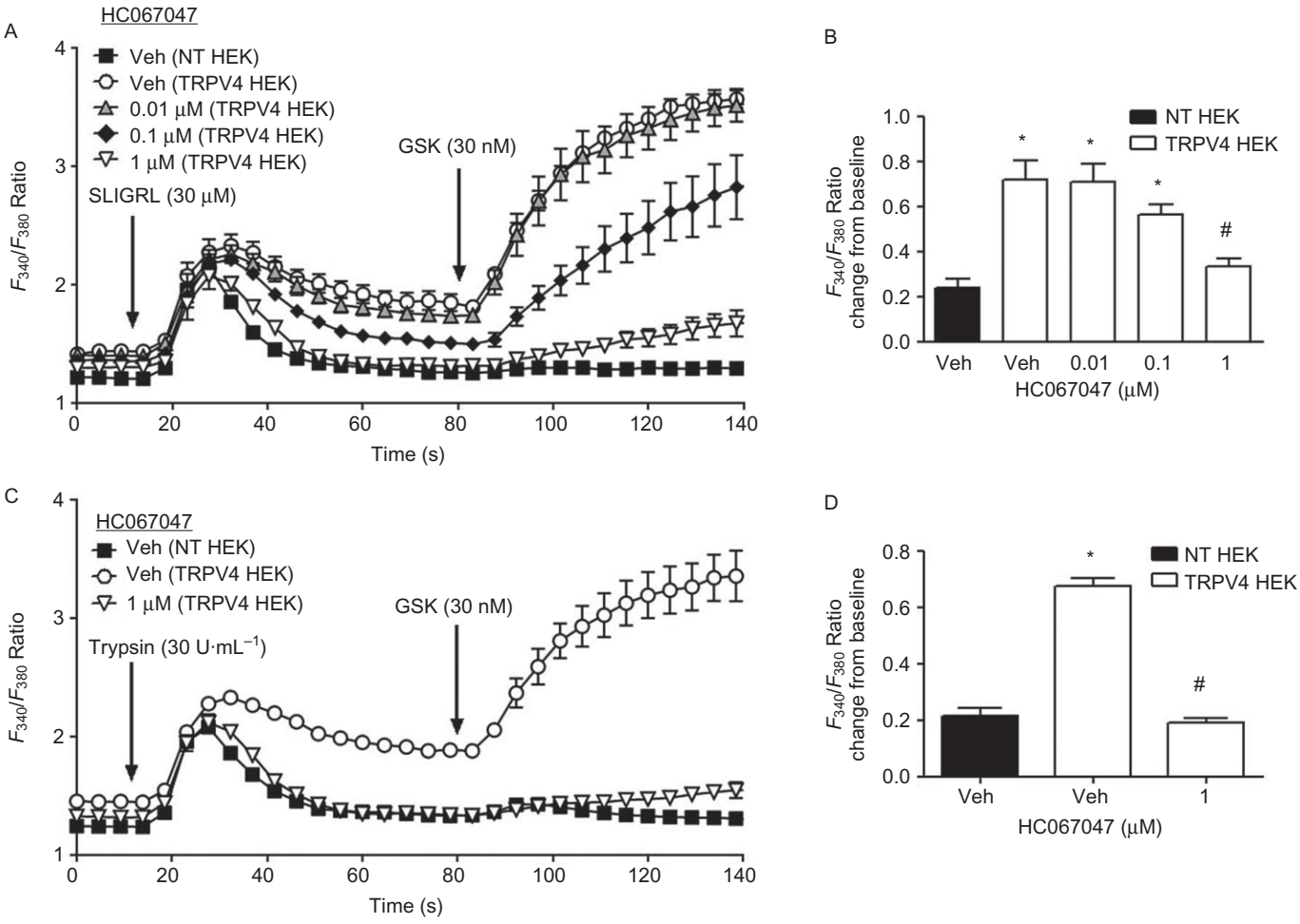

Figure 1

The sustained calcium response to PAR2 in TRPV4-transfected HEK293 cells is inhibited by TRPV4 antagonism. (A,B) The selective TRPV4 inhibitor HC067047 (10 nM to 1 μM) concentration dependently inhibited the sustained $[Ca^{2+}]_i$ response to SLIGRL (30 μM) and the response to GSK1016790A (GSK, 30 nM), but did not affect the peak response to SLIGRL. (B) Analysis showing concentration-dependent inhibition of the SLIGRL-induced coupling response with HC067047 in the TRPV4-transfected HEK293 cells. (C) 1 μM HC067047 inhibited the sustained $[Ca^{2+}]_i$ response to trypsin and the response to GSK1016790A, but did not affect the peak response to trypsin. (D) 1 μM HC067047 also inhibited trypsin-induced coupling in TRPV4 HEK cells compared with vehicle-treated (Veh) controls. Data are presented as mean ± SEM of n = 6–8 experiments. *P < 0.05, significantly different from NT HEK control. #P < 0.05, significantly different from vehicle-treated TRPV4 HEK control.

and release of intracellular calcium stores. We therefore investigated the effects of the SERCA inhibitor thapsigargin on PAR2-TRPV4 coupling. Thapsigargin (1 μM) abolished the intracellular calcium release caused by PAR2 activation, peak SLIGRL-induced PAR2 responses in thapsigargin-treated TRPV4 HEK cells were 0.27 ± 0.03 (F_{340}/F_{380} ratio) compared with 1.34 ± 0.05 for vehicle-treated TRPV4 HEK controls and 1.19 ± 0.06 for NT HEKs (P < 0.05). By contrast, coupling to TRPV4 channels was not affected (F_{340}/F_{380} ratio 1.08 ± 0.07 for thapsigargin-treated TRPV4 HEKs compared with 1.03 ± 0.02 for vehicle controls; P > 0.05) (Figure 2A,B). Removal of extracellular calcium in combination with thapsigargin abolished both PAR2- and TRPV4-mediated calcium responses. Therefore, release of intracellular calcium stores is not a stimulus for TRPV4 channel opening. However, other signalling events downstream of G-protein coupling could lead to TRPV4 channel gating. A novel $G\alpha_q$ inhibitor, UBO-QIC, has recently been described in the literature (Jacobsen *et al.*, 2013). UBO-QIC (100 nM) abolished PAR2-mediated intracellular calcium release, peak SLIGRL responses in UBO-QIC-treated TRPV4 HEK cells were 0.08 ± 0.03 (F_{340}/F_{380} ratio) compared with 1.89 ± 0.15 for vehicle-treated TRPV4 HEK controls and 2.02 ± 0.08 for NT HEKs (P < 0.05). Coupling to TRPV4 was evident but reduced compared with vehicle-treated TRPV4 HEKs (F_{340}/F_{380} ratio 0.47 ± 0.10 in UBO-QIC-treated TRPV4 HEKs compared with 1.13 ± 0.06 in vehicle-treated TRPV4 HEK controls; P > 0.05). However, coupling was significantly greater than NT HEK controls at the time point analysed (F_{340}/F_{380} ratio 0.18 ± 0.01 in NT HEK controls; P > 0.05) (Figure 2C,D). These data indicate that PAR2 coupling to $G\alpha_q$ mediates intracellular calcium release, but not activation of the TRPV4 ion channel. Neither thapsigargin nor UBO-QIC affected direct activation of TRPV4 channels by GSK1016790A (30 nM; P > 0.05).

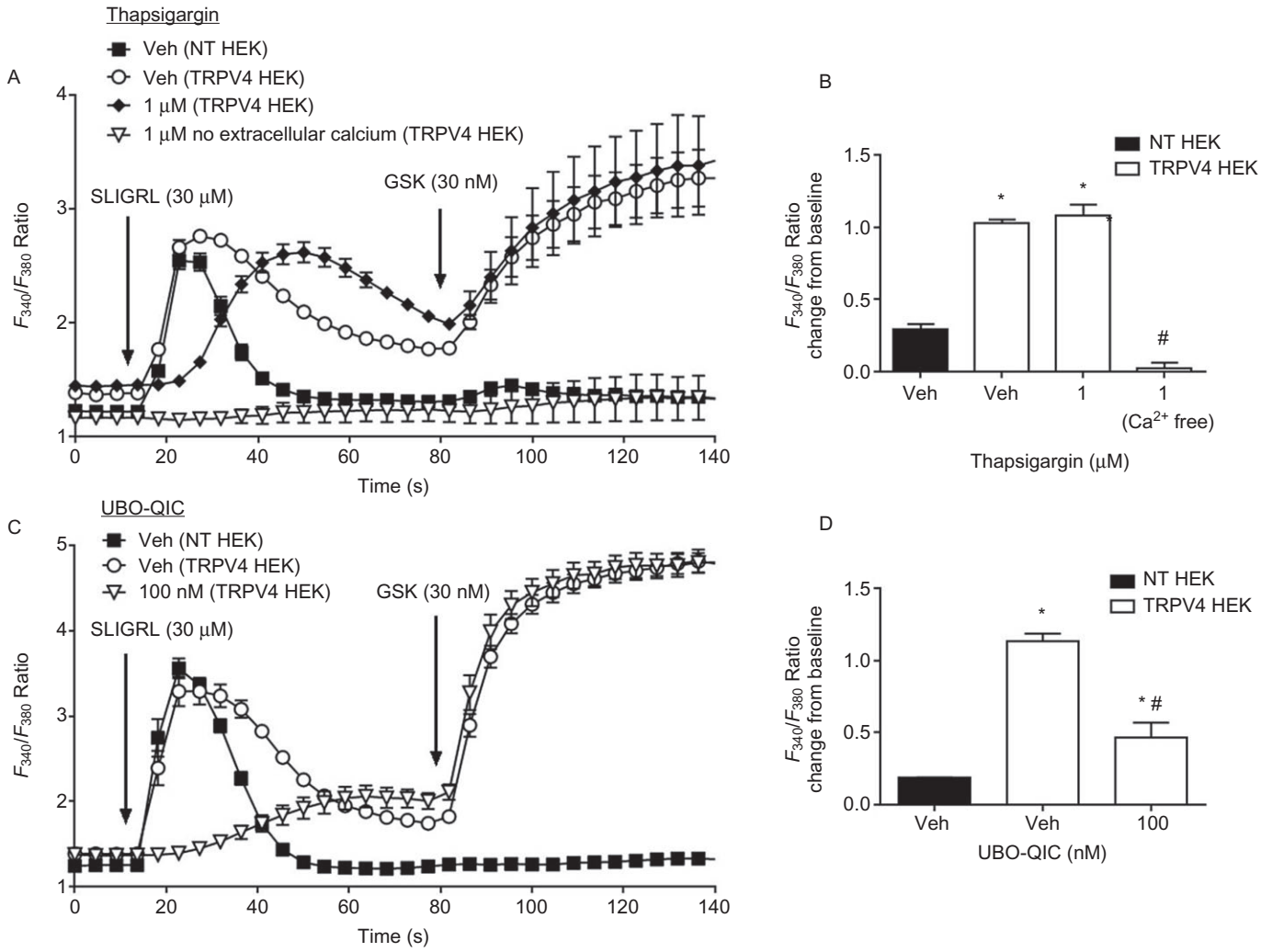

Figure 2

Gα$_q$ signalling and intracellular calcium release do not mediate PAR2-TRPV4 coupling. (A,B) the SERCA inhibitor thapsigargin (1 μM) blocked PAR2-mediated intracellular calcium release, but not influx of extracellular calcium through TRPV4 ion channels. By contrast, removal of extracellular calcium plus thapsigargin abolished PAR2-mediated intracellular calcium release, TRPV4 coupling and direct activation of TRPV4 via GSK1016790A. (C,D) The Gα$_q$ inhibitor UBO-QIC blocked PAR2-mediated intracellular calcium release, and also partly reduced coupling to TRPV4 at the time point analysed. However, at this time point, coupling in the UBO-QIC-treated TRPV4 HEKs was significantly greater than in NT HEK controls. Moreover, coupling appears to develop over time, and was equivalent to vehicle-treated TRPV4 HEK controls by approximately 55 s. Data are presented as mean ± SEM of $n = 4$ experiments. *$P < 0.05$, significantly different from NT HEK control. #$P < 0.05$, significantly different from vehicle-treated TRPV4 HEK control.

The tyrosine kinase inhibitor bafetinib blocks PAR2-TRPV4 coupling

We have previously established that the tyrosine phosphorylation site Y110 is important for PAR2-TRPV4 coupling (Poole *et al.*, 2013). This suggests that the activation of one or more tyrosine kinases is an important step in the signalling pathway leading to PAR2-TRPV4 coupling. We have further explored this finding using inhibitors selective for different tyrosine kinases. Bafetinib (1–10 μM) concentration dependently inhibited PAR2-TRPV4 coupling. In TRPV4 HEKs, 10 μM bafetinib significantly inhibited the coupling response to SLIGRL (F_{340}/F_{380} ratio 0.39 ± 0.04) and trypsin (0.52 ± 0.06) compared with vehicle control (0.66 ± 0.06 and 1.01 ± 0.11 respectively) ($P < 0.05$) (Figure 3). Conversely, bafetinib did not affect peak PAR2 or GSK1016790A responses ($P > 0.05$). Thus, bafetinib inhibits the signalling pathway leading to TRPV4 channel opening, downstream of PAR2 activation, most likely by blocking the activation of a tyrosine kinase. By contrast, dasatinib (1–10 μM) had no effect on PAR2-TRPV4 coupling at any concentration tested (SLIGRL F_{340}/F_{380} ratio 10 μM dasatinib 0.64 ± 0.02 compared with vehicle control 0.63 ± 0.05; and trypsin 10 μM dasatinib 1.96 ± 0.05 compared with vehicle 1.97 ± 0.05) ($P > 0.05$), and also did not affect PAR2 agonist or GSK1016790A peak responses ($P > 0.05$) (Figure 4).

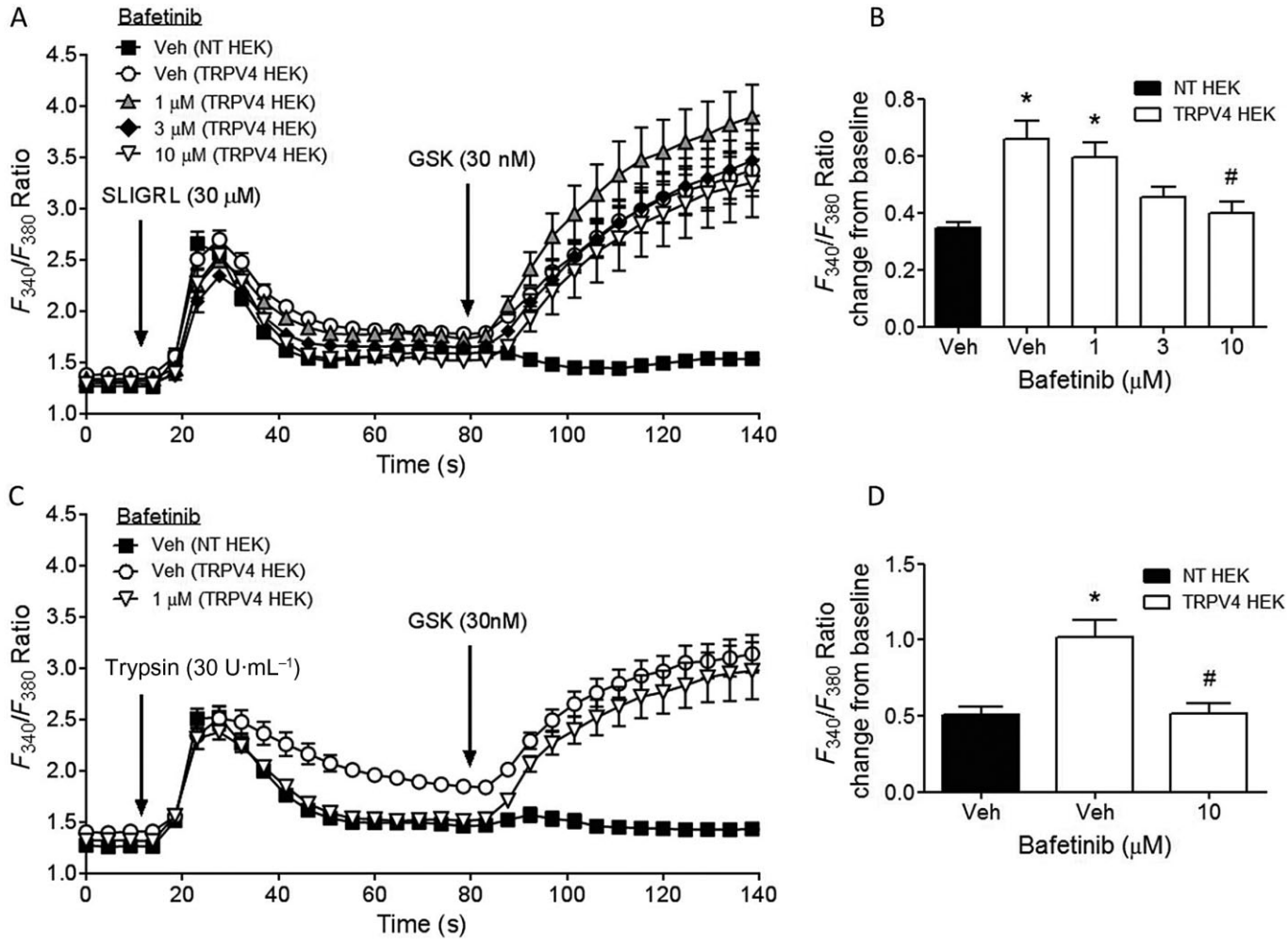

Figure 3

The tyrosine kinase inhibitor bafetinib blocks PAR2-TRPV4 coupling. (A) Bafetinib (1–10 μM) concentration dependently inhibited the sustained $[Ca^{2+}]_i$ response to SLIGRL (30 μM), without affecting the peak response to SLIGRL or GSK1016790A (GSK, 30 nM). (B) Analysis showing concentration-dependent inhibition of the SLIGRL-induced coupling response with bafetinib in the TRPV4-transfected HEK293 cells. (C) 10 μM bafetinib inhibited the sustained $[Ca^{2+}]_i$ response to trypsin, but did not affect the peak response to trypsin or the response to GSK1016790A. (D) 10 μM bafetinib inhibited trypsin-induced coupling in TRPV4 HEK cells compared with vehicle-treated (Veh) controls. Data are presented as mean ± SEM of $n = 6–7$ experiments.*$P < 0.05$, significantly different from NT HEK control. #$P < 0.05$, significantly different from vehicle-treated TRPV4 HEK control.

Inhibition of PI3Ks does not block PAR2-TRPV4 coupling

It has been previously suggested that PI3Ks are important downstream mediators of PAR2-induced TRPV4 channel stimulation in the airways (Li *et al.*, 2011). We therefore investigated a role for PI3Ks in PAR2-TRPV4 coupling. Wortmannin concentration dependently inhibited PAR2-TRPV4 coupling. In TRPV4 HEKs, 10 μM wortmannin significantly inhibited the coupling response to SLIGRL (F_{340}/F_{380} ratio 0.23 ± 0.03) and trypsin (0.61 ± 0.21) compared with vehicle control (1.07 ± 0.14 and 1.64 ± 0.17 respectively) ($P < 0.05$) (Figure 5). Conversely, wortmannin did not affect peak PAR2 or GSK1016790A responses ($P > 0.05$). However, we subsequently found that the more selective PI3K inhibitor LY294002 did not affect PAR2-TRPV4 coupling at 10 or 50 μM (SLIGRL F_{340}/F_{380} ratio 50 μM LY294002 1.35 ± 0.17 compared with vehicle 1.55 ± 0.16) ($P > 0.05$) (Figure 6A,B), indicating that PI3 kinase is unlikely to be the target of wortmannin. Wortmannin is also known to inhibit ERK1/2 activation, with an IC_{50} of approximately 1 μM (Capodici *et al.*, 1998). We therefore tested the MEK1/2 inhibitor U0126, which decreases ERK1/2 phosphorylation. U0126 did not affect coupling at 1 or 10 μM (SLIGRL F_{340}/F_{380} ratio 10 μM U0126 1.39 ± 0.22 compared with vehicle 1.36 ± 0.19) ($P > 0.05$), suggesting that wortmannin is not acting via this pathway either (Figure 6C,D).

Bafetinib inhibits PAR2-induced inflammatory pain

We have previously shown that the intraplantar injection of PAR2 agonists causes a TRPV4-dependent mechanical

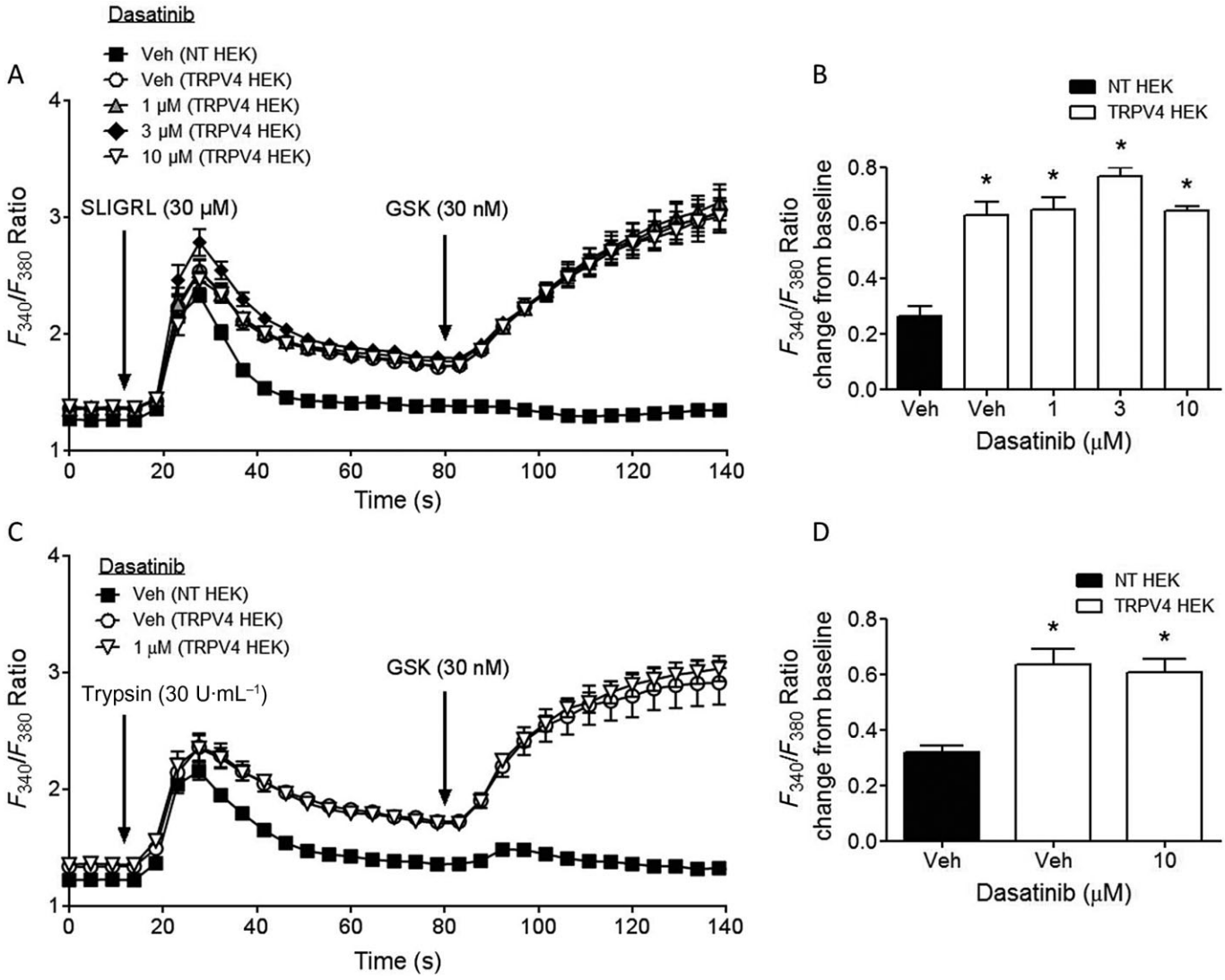

Figure 4

The tyrosine kinase inhibitor dasatinib does not block PAR2-TRPV4 coupling. (A) Dasatinib (1–10 μM) did not inhibit the sustained $[Ca^{2+}]_i$ response to SLIGRL (30 μM) at any concentration tested, and was also without effect on the peak response to SLIGRL or GSK1016790A (GSK, 30 nM). (B) Dasatinib did not inhibit SLIGRL-induced coupling in TRPV4 HEK cells. (C) 10 μM dasatinib did not inhibit the sustained $[Ca^{2+}]_i$ response to trypsin, or the peak response to PAR2 or GSK1016790A stimulation. (D) 10 μM dasatinib did not inhibit trypsin-induced coupling in TRPV4 HEK cells. Data are presented as mean ± SEM of $n = 8$ experiments. *$P < 0.05$, significantly different from NT HEK control.

hypersensitivity in mice. To investigate whether tyrosine kinase activity mediates this hypersensitivity, we treated C57BL/6 mice with vehicle (1% DMSO plus Cremophor EL), bafetinib or dasatinib (10 mg·kg^{-1}) by oral gavage 30 min prior to intraplantar injection of the PAR2 agonist SLIGRL into the left hind paw. Both compounds have been shown to be effective in mice at this dose (Shah *et al.*, 2004; Imam *et al.*, 2013; Karim *et al.*, 2013). von Frey mechanical pain threshold was subsequently measured at 0.5, 1, 2, 3 and 4 h after paw injection (for both the treated and non-treated paws). PAR2 activation caused marked mechanical hyperalgesia in the left paw of the vehicle-treated animals. Compared with vehicle controls, pretreatment of mice with bafetinib inhibited PAR2-induced mechanical hyperalgesia at all time points ($P < 0.05$) (Figure 7A). In contrast, dasatinib had no effect on mechanical hyperalgesia (Figure 7A). Unexpectedly, bafetinib also inhibited the GSK1016790A-mediated hyperalgesic response compared with controls ($P < 0.05$; Figure 6C). There was a small algesic effect observed in the untreated paw for all groups at the 3 and 4 h time points (Figure 7B,D). This may be attributable to prolonged placement of the mice on the wire mesh, and there was no difference between groups ($P > 0.05$).

Discussion

In this study, we report that the tyrosine kinase inhibitor bafetinib concentration dependently inhibits PAR2-TRPV4 coupling *in vitro*. Bafetinib also blocks PAR2-induced

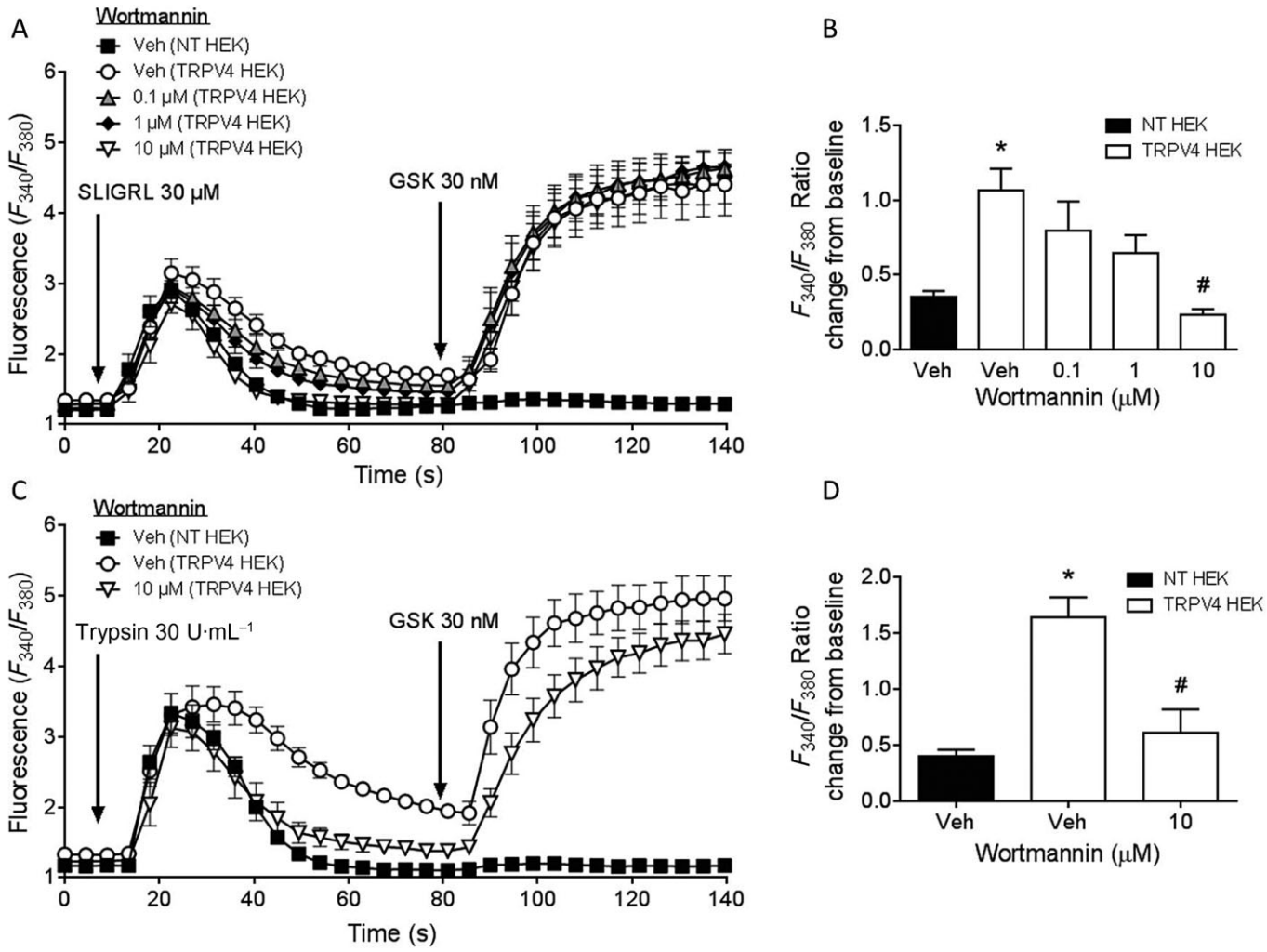

Figure 5

The PI3 kinase inhibitor wortmannin blocks PAR2-TRPV4 coupling. (A) Wortmannin (1–10 μM) concentration dependently inhibited the sustained $[Ca^{2+}]_i$ response to SLIGRL (30 μM), without affecting the peak response to SLIGRL or GSK1016790A (GSK, 30 nM). (B) Analysis showing concentration-dependent inhibition of the SLIGRL-induced coupling response with wortmannin in the TRPV4-transfected HEK293 cells. (C) 10 μM wortmannin inhibited the sustained $[Ca^{2+}]_i$ response to trypsin, but did not affect the peak response to trypsin or the response to GSK1016790A. (D) 10 μM bafetinib inhibited trypsin-induced coupling in TRPV4 HEK cells compared with vehicle-treated (Veh) controls. Data are presented as mean ± SEM of $n = 7$ experiments. *$P < 0.05$, significantly different from NT HEK control. #$P < 0.05$, significantly different from vehicle-treated TRPV4 HEK control.

mechanical hyperalgesia *in vivo*, which is known to be mediated via activation of TRPV4 (Grant *et al.*, 2007). Our studies identify a physiological function for PAR2-TRPV4 coupling, and demonstrate that hyperalgesia can be reversed by blocking intracellular signalling pathways that lead to TRP channel activation. These studies have implications for the development of novel therapeutics, as directly targeting TRP ion channels has previously led to adverse events. For example, blocking TRPV1 channels in clinical trials was shown to cause hyperthermia (Gavva *et al.*, 2008).

The signalling mechanisms leading to PAR2-TRPV4 coupling *in vitro* were investigated in the HEK293 cell line using pharmacological tools that selectively activate or inhibit specific molecular events. A role for TRPV4 ion channels in the sustained calcium response observed in TRPV4-expressing HEKs was confirmed using the selective inhibitor HC067047, which concentration dependently blocked both the sustained $[Ca^{2+}]_i$ elevation stimulated by PAR2 activation, and the subsequent response to the selective TRPV4 agonist GSK1016790A. We subsequently established that PAR2 coupling to TRPV4 is not mediated by $G\alpha_q$ signalling. $G\alpha_q$ couples to PLC, leading to IP_3-mediated release of intracellular calcium stores and the activation of second messenger kinases. The SERCA inhibitor thapsigargin and a selective $G\alpha_q$ inhibitor (UBO-QIC) both abolished the initial intracellular calcium release caused by PAR2 activation, but neither of these compounds prevented the subsequent extracellular calcium influx through TRPV4 ion channels.

We have previously shown that mutation of the tyrosine phosphorylation site Y110 reduces the PAR2-TRPV4 coupling

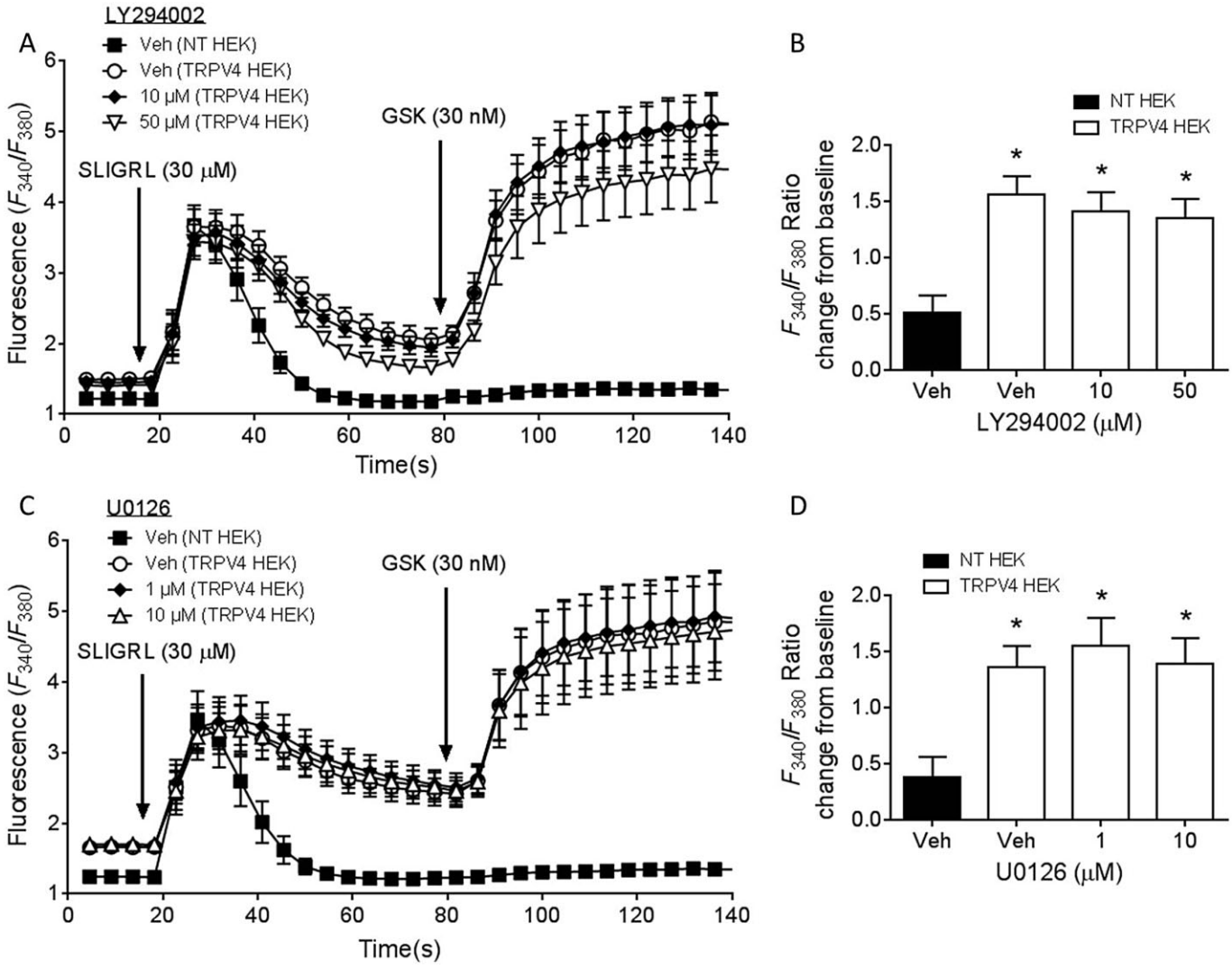

Figure 6

The selective PI3 kinase inhibitor LY294002 and the MEK1/2 inhibitor U0126 do not block PAR2-TRPV4 coupling. (A) LY294002 (10–50 μM) did not inhibit the sustained $[Ca^{2+}]_i$ response to SLIGRL (30 μM) at any concentration tested, and was also without effect on the peak response to SLIGRL or GSK1016790A (GSK, 30 nM). (B) LY294002 did not inhibit SLIGRL-induced coupling in TRPV4 HEK cells. (C) U0126 (1–10 μM) did not inhibit the sustained $[Ca^{2+}]_i$ response to SLIGRL (30 μM) at any concentration tested, and was also without effect on the peak response to SLIGRL or GSK1016790A (GSK, 30 nM). (D) U0126 did not inhibit SLIGRL-induced coupling in TRPV4 HEK cells. Data are presented as mean ± SEM of n = 4–6 experiments. *P < 0.05, significantly different from NT HEK control.

response, and small inhibitory effects of blocking selected intracellular signalling pathways (PLA_2, cytochrome P450 epoxygenase and Src kinases) on PAR2-TRPV4 coupling (Poole *et al.*, 2013). However, concentrations used for the PLA_2 inhibitor were high [1 and 10 μM methyl arachidonyl fluorophosphonate (MAFP)], and in addition to inhibiting the coupling effect also reduced PAR2-mediated intracellular calcium release and direct activation of TRPV4 channels by the selective activator GSK1016790A. Moreover, no inhibitory effect was observed for coupling at lower concentrations. This indicates that, at these concentrations, MAFP was not selective for PLA_2 activity. The cytochrome P450 epoxygenase inhibitor 17-ODYA had a small inhibitory effect on PAR2-TRPV4 coupling, but also slightly reduced the effect of 4α-PDD (another TRPV4 agonist), and thus these data are difficult to interpret. By contrast, the Src family kinase inhibitor Src1 leads to a small but significant inhibition of PAR2-TRPV4 coupling, without affecting the transient phase of PAR2 intracellular calcium release. These data support our assertion that tyrosine kinases are important in PAR2-TRPV4 coupling and, in part, implicate a role for Src kinases. However, it is likely that more than one kinase is involved.

There are over 600 known kinases in the human kinome, of which more than 60 are tyrosine kinases. Moreover, the available inhibitors are poorly selective. Investigating a role for each tyrosine kinase was not feasible for the scope of this study. We therefore wanted to test clinically relevant, broad spectrum tyrosine kinase inhibitors to establish a physiologi-

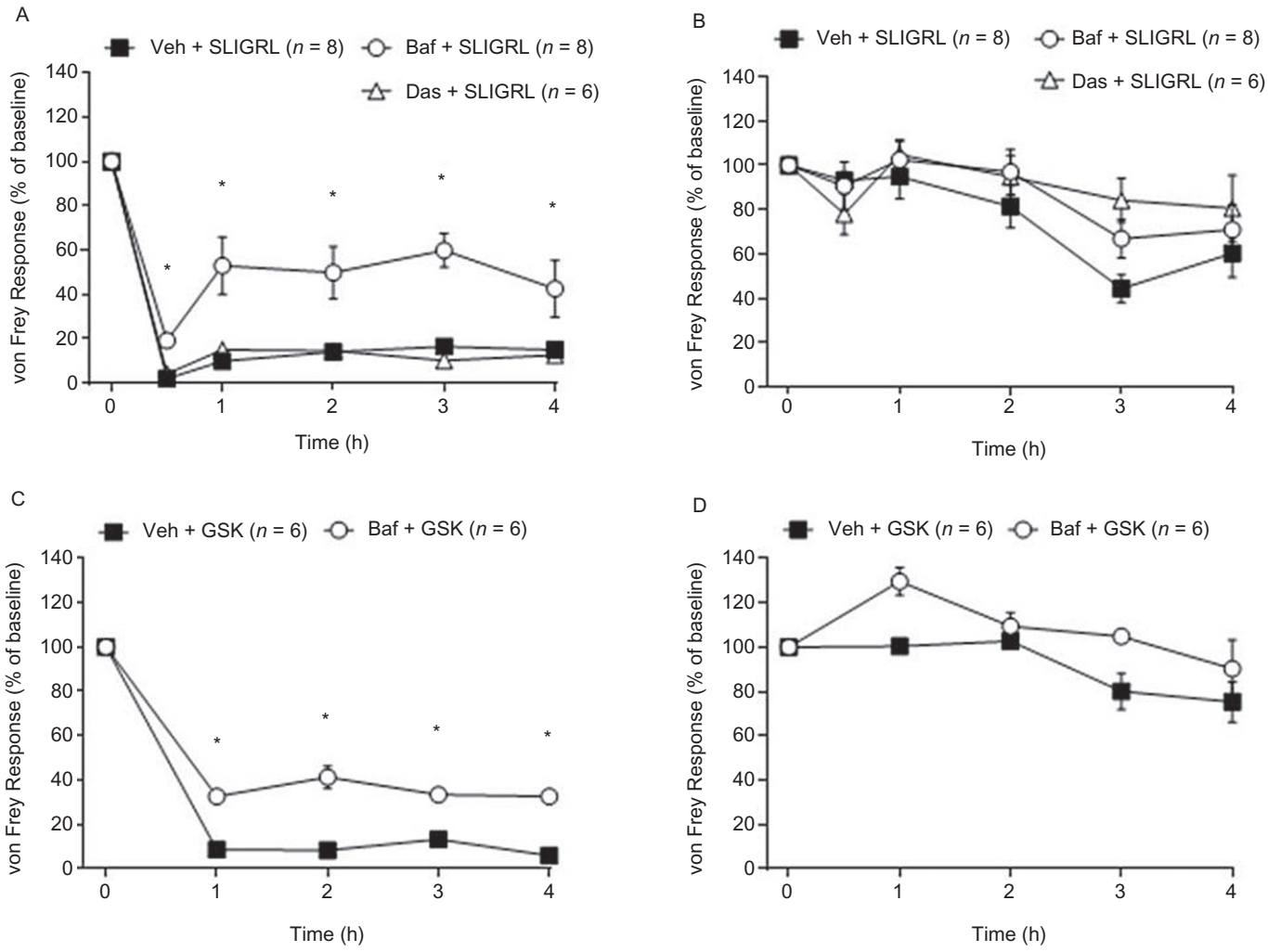

Figure 7

Effects of tyrosine kinase inhibitors on mechanical hyperalgesia in mice. Mice were pretreated with bafetinib (Baf), dasatinib (Das) or vehicle (Veh) by oral gavage. After 30 min, SLIGRL-NH2 (A,B) or GSK1016790A (GSK, C,D) were injected into one hind paw. Mechanical hyperalgesia was assessed in the injected (A,C) and non-injected (B,D) paws for 0–4 h. Data are presented as mean ± SEM of n = 6–8 experiments. *$P < 0.05$, significantly different from time-matched vehicle control.

cally and clinically applicable effect of this pathway. Dasatinib and bafetinib are new-generation anti-cancer treatments developed from the small-molecule inhibitor imatinib (Gleevec), which was originally found to produce clinical remissions in chronic myeloid leukaemia patients by inhibiting BCR-ABL (Shah *et al.*, 2004; see also Alexander *et al.*, 2013c). The development of resistance to imatinib in these patients initiated a search for similar efficacious compounds through which dasatinib and more recently bafetinib were developed (Shah *et al.*, 2004; Takeuchi *et al.*, 2009; Santos *et al.*, 2010). Having successfully completed phase I clinical trials, bafetinib is now in phase II development for leukaemia (ClinicalTrials Identifier NCT01144260) and brain tumours (ClinicalTrials Identifier NCT01234740). Here, we have shown that bafetinib concentration dependently inhibits PAR2-TRPV4 coupling in HEK293 cells, without affecting either the initial PAR2-induced calcium peak or the subsequent TRPV4 response to its selective agonist GSK1016790A. These data indicate that bafetinib is blocking the intracellular signalling pathway between PAR2 activation and TRPV4 channel gating, likely by stopping the activation of one or more tyrosine kinases. Conversely, dasatinib had no effect on the coupling response. Dasatinib has been shown to block BCR-ABL, Lyn, Src, Yes and c-KIT kinases, whereas bafetinib is known to block BCR-ABL, Lyn, Src, Lck and Fyn kinases. This could implicate Lck and/or Fyn kinases in PAR2-mediated activation of TRPV4. However, the full range of kinase targets for bafetinib and dasatinib is not known, and therefore a role for other tyrosine kinases cannot be ruled out. Kinase expression in DRG neurons and other cell types relevant to the pain pathway are poorly characterized, therefore further experiments are required to identify the bafetinib-sensitive kinases that mediate PAR2-induced activation of TRPV4. This would require a different investigative approach, such as an siRNA screen, to comprehensively determine the individual kinase(s) involved.

It has been previously suggested that $G\alpha_{i/o}$ signalling, involving the activation of PLCβ_3 and PI3Ks, is an important downstream mediator of PAR2-induced TRPV4 channel stimulation in the airways (Li *et al.*, 2011). We therefore investigated a role for PI3Ks in PAR2-TRPV4 coupling, which can be activated by GPCRs and receptor tyrosine kinases. The PI3K inhibitor wortmannin showed a significant inhibition of the PAR2-TRPV4 coupling response, without effect on PAR2-mediated intracellular calcium release or direct activation of TRPV4 channels by GSK1016790A. However, high concentrations of wortmannin were required, and the more selective PI3K inhibitor LY294002 subsequently did not inhibit PAR2-TRPV4 coupling. The effect of wortmannin is therefore likely to be off-target. Wortmannin is also known to inhibit ERK1/2 activation, with an IC_{50} of approximately 1 μM (Capodici *et al.*, 1998). We therefore tested the MEK1/2 inhibitor U0126, which decreases ERK1/2 phosphorylation, and observed no effect on PAR2-TRPV4 coupling. Therefore, wortmannin is able to inhibit PAR2-TRPV4 coupling at high concentrations via an unidentified, off-target effect.

PAR2-induced activation of peripheral and central mechanisms contributes to painful inflammation and the development of mechanical hyperalgesia, which has been shown to be mediated via sensitization of the TRPV4 ion channel (Grant *et al.*, 2007). Sensitization refers to the priming or shift in sensitivity of an ion channel to make it more likely to open with subsequent stimulation by an agonist or environmental stimuli (e.g. thermal, pH). The mechanisms by which GPCRs sensitize TRP channels are not well understood. It has been suggested to involve PLC-mediated cleavage of phosphatidyl inositol 4,5 bisphosphate in the plasma membrane, which releases the TRP channel from tonic inhibition, and also activates protein kinases that can lead to phosphorylation and modify channel gating. Specifically, PLCβ and PKs A, C and D have been implicated in the sensitization of TRPV4 (Grant *et al.*, 2007). However, we have previously demonstrated that the P_2Y activator ATP, which also couples to the $G\alpha_{q/11}$ receptor and signals via PLC, does not couple to TRPV4, and that a PKC inhibitor does not block PAR2-TRPV4 coupling (Poole *et al.*, 2013). These data indicate that the signalling pathway downstream of PAR2 activation that leads to opening of TRPV4 channels is separate from that which leads to TRPV4 sensitization.

The signalling pathway leading to TRPV4 channel opening is likely to prolong cell activation and amplify the pro-inflammatory and pro-nociceptive actions of PAR2. Using an *in vivo* model of mechanical hyperalgesia, we have shown that bafetinib (but not dasatinib) significantly inhibits PAR2-induced pain responses. Unexpectedly, bafetinib also significantly inhibited mechanical pain responses due to activation of TRPV4 channels with the selective agonist GSK1016790A. This result requires further investigation, but based on our *in vitro* data, bafetinib is unlikely to be having a direct inhibitory effect on TRPV4. It is possible that bafetinib is acting as a general analgesic. However, GSK1016790A may also be causing the release of inflammatory mediators from other cells, such as keratinocytes and endothelial cells, which signal to indirectly sensitize or activate TRPV4 channels. For example, UVB radiation has been shown to directly activate TRPV4 channels in keratinocytes, leading to increased expression of the proalgesic mediator endothelin-1, which can signal via PLC to cause further sensitization or activation of TRPV4 channels in a feedforward loop (Moore *et al.*, 2013). Therefore, bafetinib could be blocking the signalling events leading to TRPV4 channel sensitization or stimulation, and thereby inhibiting the GSK1016790A-mediated pain response. Further work needs to be done to clarify this finding.

In conclusion, we have established that bafetinib, a tyrosine kinase inhibitor that is currently in phase II clinical trials for the treatment of myeloid leukaemia, efficaciously inhibits PAR2-TRPV4 coupling *in vitro*, and PAR2-mediated mechanical hyperalgesia *in vivo*. We infer from our *in vitro* data that bafetinib is likely to be working by inhibiting the activation of tyrosine kinase(s), which stops the subsequent phosphorylation of TRPV4 channels, thereby reducing the pain response. This does not exclude the possibility that other mechanisms, such as PAR2-mediated sensitization of TRPV4 channels, are at play in the pain pathway. In fact, sensitization and coupling may be related and are likely to occur in concert, both contributing to hyperalgesia. If it is found to be safe in the current clinical trials, bafetinib could also be tested as a novel analgesic therapy for inflammatory pain.

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Author contributions

M. S. G., B. D., F. C. A. and N. V. performed the calcium experiments, and M. S. G. analysed the data. T. L. performed the *in vivo* experiments and analysed the data. P. M. and N. W. B. conceived and supervised the project. M. S. G. wrote the manuscript. All authors contributed to editing and proofreading of the manuscript.

Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Characterization of selective TRPV4 compounds in HEK293 cells. (A) The selective TRPV4 channel agonist GSK1016790A (1 nM to 1 μM) caused concentration-dependent increases in $[Ca^{2+}]_i$ in TRPV4-transfected (TRPV4 HEK) but not non-transfected (NT HEK) HEK293 cells. Vehicle (0.1% DMSO in HBSS) did not cause calcium flux in NT or TRPV4 HEKs. (B) The selective TRPV4 antagonist HC067047 (10 nM to 1 μM) concentration dependently shifted the GSK1016790A response curve in TRPV4 HEKs, in a manner consistent with a competitive antagonist. The EC_{50} for GSK1016790A was significantly shifted from 42 ± 4.8 nM (vehicle control, 0.1% DMSO) to 727 ± 21 nM, with 1 μM HC067047 (one-way ANOVA with Dunn's *post hoc* test; $P < 0.05$). Data are presented as mean ± SEM of $n = 6$ experiments.
Figure S2 Characterization of the PAR2 agonist response in HEK293 cells. Both (A) the selective synthetic tethered ligand-based activating peptide SLIGRL (100 nM to 100 μM) and (B) the non-selective endogenous PAR2 activator trypsin (0.1–100 U·mL^{-1}) caused concentration-dependent $[Ca^{2+}]_i$ increases in non-transfected (NT HEK) and TRPV4-transfected (TRPV4 HEK) HEK293 cells. No difference in the peak calcium response was observed between NT and TRPV4 HEKs. Vehicle control (0.1% dH_2O) did not elicit a calcium response in either cell type. Data are presented as mean ± SEM of $n = 6$ experiments.
Figure S3 Expression of TRPV4 leads to a sustained calcium response to PAR2 agonists in HEK293 cells. (A) 30 μM SLIGRL and (B) 30 U·mL^{-1} trypsin cause a transient increase in $[Ca^{2+}]_i$ in NT HEKs. Expression of the TRPV4 ion channel (TRPV4 HEK) caused a sustained phase increase in $[Ca^{2+}]_i$. We have referred to this phenomenon as 'coupling'. The time point at which coupling in the TRPV4 HEKs was most evident (i.e. when the difference in F_{340}/F_{380} fluorescence ratio was greatest between NT and TRPV4 HEKs) was statistically analysed for the full concentration response for each agonist. (C) SLIGRL coupling was analysed at 41 s, and found to be statistically significant from NT HEKs at 10, 30 and 100 μM. (D) Trypsin coupling was analysed at 46 s, and found to be statistically significant from NT HEKs at 10, 30 and 100 U·mL^{-1}. *$P < 0.05$, significantly different from time-matched NT HEK control. Data are presented as mean ± SEM of $n = 6$ experiments.