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Sensitisation of TRPV4 by PAR_2 is independent of intracellular calcium signalling and can be mediated by the biased agonist neutrophil elastase

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Abstract

Proteolytic activation of protease-activated receptor 2 (PAR_2) may represent a major mechanism of regulating the transient receptor potential vanilloid 4 (TRPV4) non-selective cation channel in pathophysiological conditions associated with protease activation (e.g. during inflammation). To provide electrophysiological evidence for PAR_2-mediated TRPV4 regulation, we characterised the properties of human TRPV4 heterologously expressed in *Xenopus laevis* oocytes in the presence and absence of co-expressed human PAR_2. In outside-out patches from TRPV4 expressing oocytes, we detected single-channel activity typical for TRPV4 with a single-channel conductance of about 100 pS for outward and 55 pS for inward currents. The synthetic TRPV4 activator GSK1016790A stimulated TRPV4 mainly by converting previously silent channels into active channels with an open probability of nearly one. In oocytes co-expressing TRPV4 and PAR_2, PAR_2 activation by trypsin or by specific PAR_2 agonist SLIGRL-NH_2 potentiated

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the GSK1016790A-stimulated TRPV4 whole-cell currents several fold, indicative of channel sensitisation. Pre-incubation of oocytes with the calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA)-AM did not reduce the stimulatory effect of PAR_2 activation on TRPV4, which indicates that the effect is independent of intracellular calcium signalling. Neutrophil elastase, a biased agonist of PAR_2 that does not induce intracellular calcium signalling, also caused a PAR_2-dependent sensitisation of TRPV4. The Rho-kinase inhibitor Y27362 abolished elastase-stimulated sensitisation of TRPV4, which indicates that Rho-kinase signalling plays a critical role in PAR_2-mediated TRPV4 sensitisation by the biased agonist neutrophil elastase. During acute inflammation, neutrophil elastase may sensitise TRPV4 by a mechanism involving biased agonism of PAR_2 and activation of Rho-kinase.

Keywords

TRPV4; PAR_2; Proteases; Proteolytic activation; Elastase; Rho-kinase; Two-electrode voltage clamp; *Xenopus laevis* oocytes

Introduction

Transient receptor potential vanilloid 4 (TRPV4), a member of the TRP channel superfamily, has a broad spectrum of pathophysiological functions and is expressed in a wide range of tissues [20, 31, 48]. As TRPV4 can be activated by hypotonicity, it may serve as a mechanosensor or as an osmosensor [20, 39, 40]. TRPV4 also plays an important role in inflammation [7, 69, 79], where its activation can induce formation of proinflammatory mediators during osmotic stress [53]. Moreover, TRPV4 may mediate mechanical nociception and hyperalgesia [4, 8, 24, 62]. The importance of TRPV4 is reinforced by the identification of TRPV4 mutations that are associated with neurological disorders [14, 36] or with abnormalities in bone and joint function and growth [35, 37, 61].

Multiple stimuli activate TRPV4, including moderate heat (>25 °C), ultraviolet radiation [46], cell swelling, endogenous chemicals such as arachidonic acid and its metabolite 5,6-epoxyeicosatrienoic acid, and a growing number of exogenous chemical ligands [20, 48], including many which are plant-derived, e.g. phorbol esters such as 4α-phorbol 12,13-didecanoate (4α-PDD). Moreover, there are several synthetic agonists of TRPV4, including RN-1747 and GSK1016790A [30, 71]. The selective agonist GSK1016790A is 300-fold more potent than 4α-PDD [67]; however, the mechanism of TRPV4 activation by GSK1016790A is not yet understood. Antagonists of TRPV4 include ruthenium red, which is a non-selective pore blocker of several TRPV channels [48], and the selective antagonists GSK205 [53] and HC067047 [21].

TRP channels are major downstream targets of G protein-coupled receptors (GPCRs), which can activate signalling pathways that lead to altered channel gating or to the generation of channel agonists. In particular, there is good evidence that activation of protease-activated receptor-2 (PAR_2) sensitises TRPV4 [68]. For example, in human respiratory epithelial cells, diesel exhaust particles have been reported to initiate signal transduction from PAR_2 to TRPV4 [38]. Mechanisms of PAR_2-mediated sensitisation of TRPV4 include the activation of protein kinase C and the generation of endogenous agonists [24, 54, 62]. PAR_2 belongs

to the group of four protease-activated GPCRs that mediate the effects of diverse proteases on haemostasis, inflammation, pain and healing [52, 56]. Trypsin cleaves human PAR_2 at $R^{36}\downarrow S^{37}$ to expose the tethered ligand S^{37}LIGKV, which binds to and activates the cleaved receptor. Synthetic peptides that mimic this domain directly activate the receptor [3, 50]. Other proteases that cleave at this canonical site include trypsin IV [11, 32], tryptase [10, 45], coagulation factors VIIa and Xa [5], acrosin [63], granzyme A [27], membrane-type serine protease 1 or matriptase [66], TMPRSS2 [78] and kallikrein 2, 4, 5, 6 and 14 [44, 51, 58, 59]. Other proteases can cleave PAR_2 at distinct sites, which may destroy or remove the tethered ligand domain, and thereby disarm the receptor. However, recent observations suggest that cleavage at alternative sites may cause biased agonism by stabilizing distinct active conformations of the receptor and thereby activate alternative signalling pathways. Neutrophil elastase cleaves PAR_2 at $S^{68}\downarrow V^{69}$, which removes the tethered ligand, and thereby prevents trypsin-stimulated PAR_2 signalling [18, 56]. Elastase cleavage of PAR_2 also induces PAR_2-dependent activation of extracellular-signal-regulated kinases 1/2 (ERK1/2) by a Rho-kinase-dependent pathway [56] that is distinct from trypsin-induced mitogen-activated protein kinase (MAPK) activation mediated by β-arrestins [13]. Importantly, neutrophil elastase cleavage of PAR_2 does not involve intracellular calcium signalling [29, 56]. These observations suggest that neutrophil elastase is a biased agonist of PAR_2, stimulating the receptor to signal by pathways that differ from those activated by trypsin. However, the functional relevance of elastase activation of PAR_2 is unknown.

To define the molecular mechanisms by which trypsin- and elastase-activated PAR_2 can regulate TRPV4, we studied TRPV4 activation by channel agonists and proteases using the *Xenopus laevis* oocyte expression system, a powerful tool to study the function and regulation of ion channels and PARs.

Material and methods

Chemicals

GSK1016790A, HC067047, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA)-AM, trypsin I from bovine pancreas and soybean trypsin inhibitor (SBTI) were from Sigma. Human neutrophil elastase was from SERVA electrophoresis (81 U/mg); elastase inhibitor II and the selective Rho-kinase inhibitor Y27632 were from Merck. *N*-methyl-D-glucamine (NMDG) was from Fluka. GB88, 2-furoyl LIGRL-NH_2, and SLIGRL-NH_2 were from American Peptide Company, Inc. (Sunnyvale, CA, USA).

Plasmids

Complementary DNA (cDNA) for human wild-type (wt) TRPV4 was subcloned into pTLN vector. Full-length cDNA for human PAR_2 (N-terminal FLAG and C-terminal HA11 epitopes) was subcloned into pcDNA3.1. Linearised plasmids were used as templates for complementary RNA (cRNA) synthesis (mMESSAGE mMACHINE) using T7 promoter.

Isolation of oocytes and injection of cRNA

Oocytes were obtained from adult *X. laevis* in accordance with the principles of German legislation, with approval by the animal welfare officer for the Friedrich-Alexander-

University Erlangen-Nürnberg and under the governance of the state veterinary health inspectorate (permit no. 621–2531.32–05/02). The animals were anaesthetised in 0.2 % MS222. Ovarian follicles were surgically removed through a small abdominal incision. After suture, the animals were allowed to recover fully in a separate tank before they were returned to the frog colony 1 day later. Oocytes were treated with 600–700 U/ml collagenase type 2 from *Clostridium histolyticum* (CLS 2, Worthington) dissolved in a solution containing (in mM: NaCl 82.5, KCl 2, $MgCl_2$ 1 and HEPES 1, pH 7.4, with NaOH) at 19 °C for 3–4 h. The enzymatic digestion permits the isolation of the oocytes from the ovarian lobe. Defolliculated stage V–VI oocytes were injected (Nanoject II automatic injector, Drummond) with 0.5 ng of TRPV4 cRNA and 10 ng of PAR_2 cRNA. Injected oocytes were stored at 19 °C in ND96 solution (in mM: NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, pH 7.4, with Tris) supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin.

Two-electrode voltage clamp

The oocytes were studied 2 days after injection using the two-electrode voltage clamp technique as described previously [25, 60]. Oocytes were placed in a small experimental chamber and constantly superfused at room temperature with Ca^{2+}-free solution (in mM: NaCl 96, KCl 2, $MgCl_2$ 1, HEPES 5, EGTA 1, pH 7.4, with NaOH) at a flow rate of approximately 2–3 ml/min. We used a Ca^{2+}-free solution to prevent an activation of endogenous calcium-activated chloride channels by TRPV4-mediated calcium influx and to delay a calcium-induced decay of TRPV4 currents [75]. The simplistic approach of using a Ca^{2+}-free solution has certain advantages in our experimental system but ignores aspects of TRPV4 channel function and regulation that are dependent on the presence of extracellular Ca^{2+}. To prevent sodium influx, we used a Ca^{2+}-free solution in which NaCl was replaced by NMDG-Cl (in mM: NMDG-Cl 96, KCl 2, $MgCl_2$ 1, HEPES 5, EGTA 1, pH 7.4, with Tris). Oocytes were voltage-clamped at −60 mV. Bath solution and drug exchanges were controlled by a magnetic valve system (ALA BPS-8) in combination with a TIB14 interface (HEKA). Voltage-clamp experiments were performed using an OC-725C amplifier (Warner Instruments Corp.) interfaced via LIH-1600 (HEKA) to a PC with PULSE 8.67 software (HEKA) for data acquisition and analysis. To test the effect of trypsin and neutrophil elastase, individual oocytes were pre-incubated in 130 μl of a protease-containing or a protease-free solution (Ca^{2+}-free). Afterwards, the oocytes were transferred to the experimental chamber, and GSK1016790A was used to activate TRPV4.

Single-channel recordings in outside-out patches

Single-channel recordings in outside-out membrane patches of TRPV4 expressing oocytes were performed 2 days after cRNA injection essentially as described previously [15, 16, 26, 60], using conventional patch clamp technique. Patch pipettes were pulled from borosilicate glass capillaries and had a tip diameter of about 1–1.5 μm after fire polishing. Pipettes were filled with K gluconate pipette solution (in mM: K gluconate 90, NaCl 5, Mg-ATP 2, EGTA 2, HEPES 10, pH 7.28, with Tris). Seals were routinely formed in a low-sodium NMDG-Cl bath solution (in mM: NMDG-Cl 95, NaCl 1, KCl 4, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 10, pH 7.4, with Tris). In this bath solution, the pipette resistance averaged ~7.5 MΩ. After seal formation, the NMDG-Cl solution was switched to a NaCl Ca^{2+}-free bath solution

in which NMDG-Cl (95 mM) was replaced by NaCl (95 mM), $CaCl_2$ was removed, and EGTA (1 mM) was added. For continuous current recordings, membrane patches were routinely voltage-clamped at −82 mV, close to the calculated equilibrium potential of Cl^- (E_{Cl}=−77.4 mV) and K^+ (E_K=−79.4 mV) under our experimental conditions. Experiments were performed at room temperature. Single-channel current data were initially filtered at 2.5 kHz and sampled at 10 kHz. The current traces were re-filtered at 250 Hz to resolve the single-channel current amplitude (i) and channel activity. The latter was derived from binned amplitude histograms as the product NP_O, where N is the number of channels and P_O is open probability [15, 16, 33, 34]. Single-channel data were analysed using the program "Patch for Windows" written by Dr. Bernd Letz (HEKA Elektronik) and the program "Nest-o-Patch" written by Dr. V. Nesterov (Institut für Zelluläre und Molekulare Physiologie, Friedrich-Alexander-University Erlangen-Nürnberg, Erlangen, Germany). Using a 3 M KCl flowing boundary electrode, the liquid junction potential occurring at the pipette/NaCl bath junction was measured to be ~12 mV (bath positive) and was not significantly affected by the removal of divalent cations. V_{hold} values are corrected for the liquid junction potential.

Statistical methods

Data are presented as mean ± SEM. Statistical significance was assessed by appropriate version of Student's t test with GraphPad Prism 4.03 (GraphPad Software) for Windows.

Results

Functional evidence for TRPV4 expression in the oocyte expression system

To confirm functional TRPV4 expression in oocytes injected with TRPV4 cRNA, we made continuous whole-cell current recordings at a holding potential of −60 mV as illustrated in Fig. 1. The known TRPV4 agonist GSK1016790A [67] and the selective TRPV4 inhibitor HC067047 (100 nM) [21] were used as tools to activate and inhibit TRPV4 currents, respectively. The representative whole-cell current recording shown in Fig. 1a demonstrates that 50 nM GSK1016790A stimulated a substantial inward current, whereas 1 and 10 nM GSK1016790A had no effect. Washout of GSK1016790A caused a delayed and incomplete return towards the baseline current level. A subsequent application of GSK1016790A in a concentration of 100 nM elicited an even larger stimulatory response, which was partially reversible after washout of the agonist. The sustained stimulated inward current component that remained after the washout of the agonist was inhibited by HC067047, confirming that the inward current elicited by GSK1016790A is mediated by TRPV4. As expected, the non-specific TRPV4 blocker ruthenium red also inhibited the TRPV4-mediated inward current activated by GSK1016790A (data not shown). Importantly, in control experiments using non-injected oocytes, GSK1016790A did not stimulate any inward current (data not shown). We then applied GSK1016790A (50 nM) for different periods of time (30 s, 1 min and 2 min; Fig. 1b). These experiments indicate that the stimulatory effect of GSK1016790A increased with longer exposure time. Furthermore, the stimulatory response to GSK1016790A increased with repeated applications of the agonist (Fig. 1c). A similar phenomenon of increased current amplitude with repeated agonist application has been described for TRPA1 [55]. Under our experimental conditions with a holding potential of −60 mV and in the absence of extracellular calcium ions, the TRPV4 currents activated

by GSK1016790A should be carried by an influx of sodium ions. To confirm this, we studied oocytes in which extracellular NaCl was replaced by NMDG-Cl, which is too large to permeate the TRPV4 pore (Fig. 1d). After an initial inward current activation by GSK1016790A, changing the bath solution to NMDG-Cl resulted in a small reduction of the inward current back to baseline level. This is consistent with the interpretation that the inward current component that persists after application of GSK1016790A is carried by sodium ions. Importantly, in the absence of extracellular sodium ions, a second application of GSK1016790A failed to elicit an inward current. The responsiveness to GSK1016790A was restored after changing back to a sodium ion containing bath solution (Fig. 1d). These findings confirm that the inward currents observed upon GSK1016790A application are carried by the influx of sodium ions through activated TRPV4 channels. These experiments demonstrate that *X. laevis* oocytes are a suitable system to express functional TRPV4 channels and that the expressed channels can be activated reliably with GSK1016790A. To achieve comparable levels of TRPV4 activation, we applied GSK1016790A in a concentration of 50 nM for 45 s in all subsequent two-electrode voltage-clamp experiments.

Single-channel properties of TRPV4 and its activation by GSK1016790A

Single-channel properties of human TRPV4 expressed in oocytes have not yet been studied in detail. To resolve TRPV4 single-channel currents, we made patch clamp recordings in the outside-out configuration using oocytes expressing TRPV4. Typical single-channel current traces recorded at different holding potentials are shown in Fig. 2a. Interestingly, this type of spontaneous single-channel activity was observed without prior application of GSK1016790A in five out of ten successful outside-out membrane patches obtained from TRPV4 expressing oocytes but not in non-injected control oocytes (Fig. 2d). The closed channel level (−C) was determined by visual inspection of the traces. At this level, the electrical noise was significantly lower than at the channel open level (−1). The average single-channel *I-V* plot shown in Fig. 2b summarises data from similar experiments as shown in Fig. 2a. A polynomial fit of these data revealed a reversal potential of −0.5 mV (n=11) indicative of a non-selective cation channel that does not discriminate between the monovalent cations Na^+ and K^+. Under our experimental conditions, TRPV4-mediated inward currents are carried by Na^+ at hyperpolarising holding potentials, and outward currents are carried by K^+ at depolarising holding potentials. From the polynomial fit shown in Fig. 2b, we estimated the following single-channel slope conductance values: 55 pS at V_{hold}=−80 mV, 8 pS at V_{hold}=−40 mV, 30 pS at V_{hold}=+40 mV and 99 pS at V_{hold}=+80 mV (n=11). Thus, the channel displays inward as well as outward rectification with a slightly larger outward than inward conductance. These are typical features of TRPV4 [20]. The mechanism by which GSK1016790A activates TRPV4 is not known. To investigate the activation of TRPV4 by GSK1016790A at the single-channel level, we made outside-out patch clamp recordings from oocytes expressing TRPV4 (V_{hold}=−82 mV). As shown in Fig. 2c, spontaneous TRPV4 channel activity was observed with one active channel in the patch even before application of the TRPV4 activator GSK1016790A. Long bursts of channel activity (>10 s) with an open probability of nearly one alternated with periods of complete channel closure. Application of extracellular GSK1016790A caused a stepwise appearance of several additional channel levels resulting in a significant increase in the overall activity (NP_O) of TRPV4, as summarised in Fig. 2e. This significant increase in

the number of active channels in the patch (N_{active}) after application of GSK1016790A was confirmed in nine additional recordings (Fig. 2f). These single-channel data indicate that recruitment of near-silent channels largely contributes to the stimulatory effect of GSK1016790A on TRPV4. Application of GSK1016790A did not alter the single-channel current amplitude of TRPV4 (Fig. 2c). Before and after application of GSK1016790A at V_{hold}=−82 mV, the single-channel current amplitude averaged at −4.66±0.32 pA (n=5) and −4.84±0.34 pA (n=5, n.s.), respectively. Washout of GSK1016790A for ~30 s did not significantly change the single-channel activity. However, extracellular application of the TRPV4 inhibitor HC067047 decreased NP_O and N_{active} towards baseline values, confirming that the channel activity elicited by application of GSK1016790A can be attributed to TRPV4 (Fig. 2c, summary in Fig. 2e, f). In control experiments using non-injected oocytes, no comparable spontaneous single-channel activity was detected and application of both GSK1016790A and HC067047 had no detectable effect (Fig. 2d). In summary, these data demonstrate that TRPV4 expressed in oocytes functions as a non-selective cation channel with a similar single-channel conductance for Na^+ and K^+ and with an inwardly and outwardly rectifying I-V relationship typical for this channel [20]. GSK1016790A activates TRPV4 by converting previously silent channels in the excised membrane patch into active channels without changing the single-channel current amplitude.

Functional evidence for PAR_2 expression in the oocyte expression system

X. laevis oocytes are known to express endogenous calcium-activated chloride channels [2, 22]. Moreover, they may express endogenous PAR_2-like receptors since application of high concentrations of trypsin (e.g. 2 μg/ml=85 nM) to non-injected oocytes has been shown to elicit a transient inward current, probably caused by an activation of endogenous calcium-activated chloride channels as a result of receptor-mediated calcium signalling [9, 15, 19]. In the present study, we demonstrated that trypsin applied in a low concentration (8 nM) had a negligible effect on baseline currents of non-injected control oocytes (Fig. 3a). In contrast, oocytes injected with cRNA for PAR_2 consistently responded to the same concentration of trypsin (8 nM) with a large transient inward current peak. This stimulatory effect of trypsin was prevented by application of trypsin with SBTI, which indicates that the response requires proteolytic trypsin activity (Fig. 3b). Application of the PAR_2 agonists 2-furoyl-LIGRL-NH_2 or SLIGRL-NH_2, which mimic the effect of the tethered ligand released by trypsin, elicited a current response similar to that caused by trypsin (Fig. 3c). Moreover, pre-incubating PAR_2 expressing oocytes for 15 min with the non-competitive non-peptide PAR_2 antagonist GB88 [41, 64] reduced the current response to trypsin by ~60 % (Fig. 3d). This inhibition is in good agreement with the effect of GB88 on PAR_2 in HEK293 cells (data not shown). Finally, we demonstrated that pre-incubation of PAR_2 expressing oocytes with BAPTA-AM to buffer intracellular calcium abolished the current response to trypsin (Fig. 3e) and also to the PAR_2 agonists 2-furoyl-LIGRL-NH_2 or SLIGRL-NH_2 (Fig. 3f) in these oocytes. This finding is consistent with the interpretation that trypsin and the PAR_2 agonists stimulate a PAR_2-dependent current that is mediated by stimulation of calcium-activated chloride channels. Taken together, our findings indicate functional expression of PAR_2 in oocytes injected with PAR_2 cRNA.

Proteolytic activation of PAR_2 by trypsin sensitises TRPV4

To investigate a possible role of PAR_2 in TRPV4 regulation, we co-expressed TRPV4 with PAR_2. Co-expression of PAR_2 per se did not significantly alter TRPV4-mediated currents elicited by the application of GSK1016790A in a concentration of 50 nM for 45 s (TRPV4 currents averaged 1.73±0.13 μA or 1.93±0.10 μA (N=2, n=14; data not shown) in oocytes expressing TRPV4 or TRPV4/PAR_2, respectively). We pre-incubated oocytes expressing TRPV4 alone or co-expressing TRPV4 and PAR_2 for 1, 5 or 25 min with 8 nM trypsin. Subsequently, we assessed the GSK1016790A-induced TRPV4 whole-cell currents using two-electrode voltage-clamp measurements (Fig. 4). Our results show that TRPV4 currents were significantly larger in trypsin pre-treated oocytes co-expressing TRPV4 and PAR_2 compared to those in oocytes expressing TRPV4 alone. The stimulatory effect of trypsin was maximal in TRPV4/PAR_2 co-expressing oocytes treated with trypsin for 5 min (Fig. 4a, b). Importantly, we demonstrated in control experiments that the trypsin inhibitor SBTI prevented the stimulatory effect of trypsin on TRPV4 currents in TRPV4/PAR_2 co-expressing oocytes (Fig. 4c). In summary, our results indicate that sensitisation of TRPV4 currents by trypsin pre-incubation is dependent on PAR_2 co-expression and is likely to be mediated by proteolytic PAR_2 activation.

Activation of PAR_2 by PAR_2 agonists (2-furoyl-LIGRL-NH_2 or SLIGRL-NH_2) also sensitises TRPV4

In additional experiments, we investigated the effect of two known PAR_2-activating peptides (2-furoyl-LIGRL-NH_2 and SLIGRL-NH_2) on TRPV4 currents in oocytes expressing TRPV4 alone or co-expressing TRPV4/PAR_2 (Fig. 5). As illustrated in Fig. 3c, the two peptides elicited a similar transient inward current response as activation of PAR_2 by trypsin. TRPV4 and TRPV4/PAR_2 expressing oocytes were pre-incubated for 5 min in 2-furoyl-LIGRL-NH_2 (10 μM) or SLIGRL-NH_2 (100 μM). Subsequently, GSK1016790A-induced TRPV4 currents were measured (Fig. 5a). TRPV4 currents were significantly larger in TRPV4/PAR_2 expressing oocytes compared to those measured in oocytes expressing TRPV4 alone (Fig. 5b). These results indicate PAR_2-dependent sensitisation of TRPV4 by PAR_2 agonists.

Inhibition of PAR_2 by GB88 causes a reduction of TRPV4 sensitisation

To further confirm the role of PAR_2 in trypsin-induced sensitisation of TRPV4 currents in TRPV4/PAR_2 co-expressing oocytes, we studied the effect of the PAR_2 antagonist GB88. Oocytes co-expressing TRPV4/PAR_2 were pre-incubated for 15 min with GB88 (10 μM) or vehicle (control). Subsequently, oocytes were exposed to trypsin for 5 min, and the GSK1016790A-induced TRPV4 currents were measured. Inhibition of PAR_2 by GB88 reduced trypsin-induced activation of TRPV4 currents by ~70 % compared to control (Fig. 6).

PAR_2-mediated sensitisation of TRPV4 is not affected by BAPTA-AM

In control experiments, similar to those shown in Fig. 3e, we confirmed that pre-incubation in BAPTA-AM abolished the trypsin-induced activation of calcium-activated chloride currents in TRPV4/PAR_2 co-expressing oocytes (data not shown). This finding indicates that pre-incubation in BAPTA-AM prevents a PAR_2-mediated rise in intracellular calcium. In

matched control oocytes co-expressing TRPV4/PAR_2, without BAPTA-AM pre-treatment, we confirmed the PAR_2-mediated sensitisation of TRPV4 by trypsin that is not observed in oocytes expressing TRPV4 alone (Fig. 7; also see Fig. 4). Importantly, a similar sensitisation of TRPV4 currents was observed in TRPV4/PAR_2 co-expressing oocytes pretreated with BAPTA-AM. This indicates that PAR_2-mediated intracellular calcium signalling is not required for sensitisation of TRPV4 by PAR_2 activation.

Proteolytic activation of PAR_2 by the biased agonist human neutrophil elastase sensitises TRPV4

Our experiments using BAPTA-AM (see Fig. 7) suggest that calcium signalling is not involved in PAR_2-mediated sensitisation of TRPV4. In additional experiments, we tested the effect of the biased agonist neutrophil elastase (Fig. 8), which activates PAR_2 without eliciting an intracellular calcium signal [29, 56]. The experimental protocol was similar to that used to investigate the effect of trypsin (Fig. 4). We exposed oocytes to vehicle (control; a) or human neutrophil elastase (3 μM; b) for 5 min and then activated TRPV4 with GSK1016790A (50 nM). We compared the magnitude of the response to the TRPV4 agonist in oocytes expressing TRPV4 alone to that in oocytes expressing TRPV4/PAR_2. GSK1016790A-activated currents were similar in vehicle-treated control oocytes expressing TRPV4 alone or TRPV4/PAR_2 (Fig. 8a, c). Pre-treatment of oocytes expressing TRPV4 alone with neutrophil elastase did not alter the response to GSK1016790A (Fig. 8b, left; Fig. 8c). In contrast, pre-incubation of oocytes expressing TRPV4/PAR_2 with elastase for 5 min amplified the response to GSK1016790A (Fig. 8b, right; Fig. 8c). To confirm that the observed increase in $\Delta I_{GSK1016790A}$ is caused by the elastase activity of the protease preparation used, we examined the effect of an elastase inhibitor on PAR_2-mediated sensitisation of TRPV4 by neutrophil elastase. The elastase inhibitor prevents PAR_2-mediated sensitisation of TRPV4 by elastase but not by trypsin (Fig. 8d). In control experiments, pre-incubation of oocytes in elastase inhibitor alone had a negligible effect on GSK1016790A-mediated TRPV4 currents (Fig. 8d). Thus, elastase activates PAR_2, which can sensitise TRPV4. Since elastase does not evoke PAR_2-dependent intracellular calcium signals, our findings further support the conclusion that intracellular calcium signalling is not involved in PAR_2-mediated sensitisation of TRPV4.

Rho-kinase inhibitor Y27362 inhibits PAR_2-mediated sensitisation of TRPV4 by neutrophil elastase

Rho-kinase inhibitors block elastase and PAR_2-dependent p44/42 MAPK signalling [56]. Therefore, we investigated the effect of the Rho-kinase inhibitor Y27362 on PAR_2-mediated sensitisation of TRPV4. Oocytes expressing TRPV4/PAR_2 were pre-incubated in neutrophil elastase (3 μM) or trypsin (8 nM) in the absence or presence of Rho-kinase inhibitor Y27362 (10 μM). Y27362 almost completely prevented PAR_2-mediated sensitisation of TRPV4 by neutrophil elastase (Fig. 9). In contrast, Y27362 did not affect PAR_2-mediated TRPV4 sensitisation by trypsin. In control experiments, Y27362 had no effect on the GSK1016790A-mediated TRPV4 currents in TRPV4-expressing oocytes. Furthermore, to rule out the possibility that the Rho-kinase inhibitor prevents PAR_2 activation by non-specifically inhibiting the proteolytic activity of elastase, we tested the channel-activating effect of elastase in the presence or absence of Rho-kinase inhibitor on oocytes expressing

the human epithelial sodium channel (ENaC). Elastase is known to proteolytically activate ENaC [1, 28]. We demonstrated that proteolytic ENaC activation by elastase was preserved in the presence of the Rho-kinase inhibitor (data not shown). Thus, the inhibitory effect of the Rho-kinase inhibitor on the PAR_2-mediated sensitisation of TRPV4 by elastase cannot be explained by an inhibition of the proteolytic activity of elastase. Taken together, these findings indicate that the signalling pathway for PAR_2-mediated sensitisation of TRPV4 by elastase critically involves Rho-kinase.

Discussion

To our knowledge, this is the first study to demonstrate that, in addition to trypsin, the biased PAR_2 agonist elastase also sensitises TRPV4 via PAR_2 signalling. This suggests that PAR_2-mediated sensitisation of TRPV4 is independent of intracellular calcium signalling, which is further supported by our finding that buffering intracellular calcium does not prevent PAR_2-mediated TRPV4 sensitisation. Moreover, we identified Rho-kinase as one of the principal signalling pathways involved in PAR_2-dependent sensitisation of TRPV4 by neutrophil elastase. In addition, we report that the stimulatory effect of the known TRPV4 channel activator GSK1016790A results from the recruitment of near-silent channels in the plasma membrane. Upon exposure to GSK1016790A, these near-silent channels are converted to channels with an open probability of nearly one.

In single-channel current recordings from outside-out patches, we confirmed that human TRPV4 functions as a non-selective cation channel permeable for sodium and potassium ions. The single-channel conductance of about 100 pS for outward currents and 55 pS for inward currents and the small degree of inward and outward rectification in our experiments are in good agreement with previously reported data. Indeed, in recent years, several groups have made TRPV4 single-channel recordings showing inward and outward rectification mainly using mouse and rat TRPV4. Watanabe et al. have reported a single-channel conductance of about 100 pS for outward currents and 60 pS for inward currents in HEK293 cells transfected with mouse TRPV4 [74, 76, 77]. In the oocyte expression system, single-channel recordings have been described for rat TRPV4 (100 pS for outward currents and 45 pS for inward currents) [42]. A higher single-channel conductance of 310 pS has been reported for chicken VR-OAC (TRPV4) expressed in CHO cells [40]. However, this value was estimated under different experimental conditions from one inside-out patch clamp recording at a holding potential of +80 mV without analysis of the current voltage relationship. For the human heteromeric TRPV4/TRPC1 channel, a single-channel conductance of about 90 pS for outward currents and 60 pS for inward currents was reported in HEK293 cells using cell-attached patches [43].

In 50 % of outside-out membrane patches from TRPV4 expressing oocytes, we observed a sizeable spontaneous single-channel activity without prior application of GSK1016790A (Fig. 2d). In contrast, spontaneous TRPV4 activity prior to the application of GSK1016790A was minimal in whole-cell current measurements in TRPV4 expressing oocytes (e.g. Fig. 1). The latter finding is expected since our experiments were performed at room temperature and TRPV4 is known to be spontaneously active only at temperatures >24 to 27 °C [20]. Thus, TRPV4 is probably inactive in oocytes maintained at room temperature but may have

some degree of spontaneous activity in mammalian cells at 37 °C. At present, the molecular mechanism of heat activation remains unclear but may involve additional heat-sensitive proteins associated with the channel in the plasma membrane [72, 73]. Therefore, the sensitivity of TRPV4 to heat may be altered by patch excision [49], which may explain our finding of spontaneous TRPV4 activity in outside-out patches from oocytes expressing TRPV4.

The selective TRPV4 agonist GSK1016790A has been widely used to study the role of TRPV4 channels [23, 67]. However, the mechanism by which GSK1016790A activates TRPV4 is not well understood. In the present study, we used the outside-out configuration of the patch clamp technique to observe the effect of GSK1016790A at the single-channel level. Analysis of our data obtained in the outside-out configuration of the patch clamp technique revealed that the activation of TRPV4 by GSK1016790A was mainly mediated by recruitment of previously silent channels that are already present in the plasma membrane and not by an increase in single-channel current amplitude. As our recordings were performed in outside-out patches under calcium-free conditions on the extracellular and intracellular sides of the plasma membrane, it is unlikely that the recruitment of additional channels was mediated by insertion of vesicles containing TRPV4, because vesicle fusion with the plasma membrane usually requires a calcium signal. Our findings provide direct electrophysiological evidence to support the conclusion of a recent study using total internal reflection fluorescence microscopy to record TRPV4 channel activity in primary human endothelial cells. The authors report that TRPV4 protein is evenly distributed throughout the plasma membrane, but that most channels are silent. Moreover, their optical recordings indicate that GSK1016790A acts by recruiting previously inactive TRPV4 channels rather than by increasing elevation of basal activity [65]. GSK1016790A may activate the silent TRPV4 channels by changing their conformation possibly by interacting with a specific binding site. However, further studies are needed to elucidate the precise molecular mechanism(s) of TRPV4 activation by GSK1016790A and the physiological role of an apparently large pool of near silent channels in the plasma membrane.

It is well known that TRPV4 mediates neurogenic inflammation and pain [47]. TRPV4 is also discussed as a potential therapeutic target to treat intestinal inflammation and inflammatory bowel disease [69]. One major downstream target of PAR_2 is TRPV4 [6, 24, 54, 62]. After activation, PAR_2 can regulate multiple pathophysiological processes, including inflammation, pain, haemostasis and wound healing. For instance, it has been reported that PAR_2 activation by trypsin causes widespread inflammation [70]. PAR_2 is also believed to play a role in allergic lung inflammation, and PAR_2 antagonists may be useful drugs to treat inflammatory airway disease [12]. Furthermore, PARs may be involved in neurogenic inflammation, in neurodegenerative processes and also in nociception [70]. Here, we demonstrated for the first time that proteolytic activation of PAR_2 by neutrophil elastase potentiates the response to a TRPV4 agonist leading to a sensitisation, which may play an important role in the context of nociception. The “coupling” of TRPV4/PAR_2 may be relevant in inflammatory diseases such as arthritis or inflammatory bowel disease, where multiple PAR_2-activating proteases are produced [24]. The observed PAR_2-mediated TRPV4 sensitisation by neutrophil elastase suggests a potential pathophysiological link between PAR_2 and TRPV4 in the context of inflammation. Elastase released from neutrophils

has been correlated to the pathologic processes of a variety of inflammatory diseases, including idiopathic pulmonary fibrosis, rheumatoid arthritis, adult respiratory distress syndrome and cystic fibrosis [17]. Activation of PAR_2 by elastase may explain the recently reported activation of TRPV4 by ultraviolet radiation, which depends on an up-regulation of neutrophil elastase in response to ultraviolet exposure of the skin [46].

In summary, our study demonstrates that the oocyte expression system is suitable to study the mechanisms of TRPV4 activation by GSK1016790A at the single-channel level and to investigate the regulation of TRPV4 by PAR_2 in co-expression experiments using different PAR_2 agonists. In whole-cell recordings, we confirmed previous findings that trypsin-mediated activation of PAR_2 markedly enhanced TRPV4 currents. Moreover, we demonstrated for the first time that the biased PAR_2 agonist neutrophil elastase could also sensitise TRPV4 in a PAR_2-dependent manner. PAR_2 signalling elicited by neutrophil elastase does not involve intracellular calcium signalling [29, 57]. This is in good agreement with our finding that PAR_2-mediated sensitisation of TRPV4 was unaffected by buffering intracellular calcium by BAPTA. Thus, our BAPTA experiments and the fact that neutrophil elastase is a biased PAR_2 agonist without calcium signalling indicate that PAR_2-mediated TRPV4 activation is independent of intracellular calcium. Our finding that neutrophil elastase causes a PAR_2-dependent sensitisation of TRPV4 reveals, for the first time, a potential physiological consequence of biased agonism of PAR_2 by elastase. Moreover, we identified Rho-kinase as one of the principal signalling pathways involved in PAR_2-mediated TRPV4 sensitisation by neutrophil elastase. Interestingly, PAR_2-mediated TRPV4 sensitisation by trypsin was not affected by inhibition of Rho-kinase. This is in good agreement with the concept that the biased PAR_2 agonist elastase activates a more limited number of signalling pathways than trypsin. Our results indicate that PAR_2-mediated TRPV4 sensitisation by trypsin does neither depend on calcium signalling nor on Rho-kinase signalling but involves another signalling pathway that remains to be elucidated. Further studies are required to determine whether elastase-induced activation of PAR_2 and sensitisation of TRPV4 could contribute to the proinflammatory and nociceptive effects of neutrophil elastase. In this case, inhibition of Rho-kinases may become a therapeutic strategy to prevent PAR_2-mediated TRPV4 sensitisation by elastase in inflammatory diseases.

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Abbreviations

TRPV4	Transient receptor potential vanilloid 4
PAR_2	Protease-activated receptor 2
GPCR	G protein-coupled receptor

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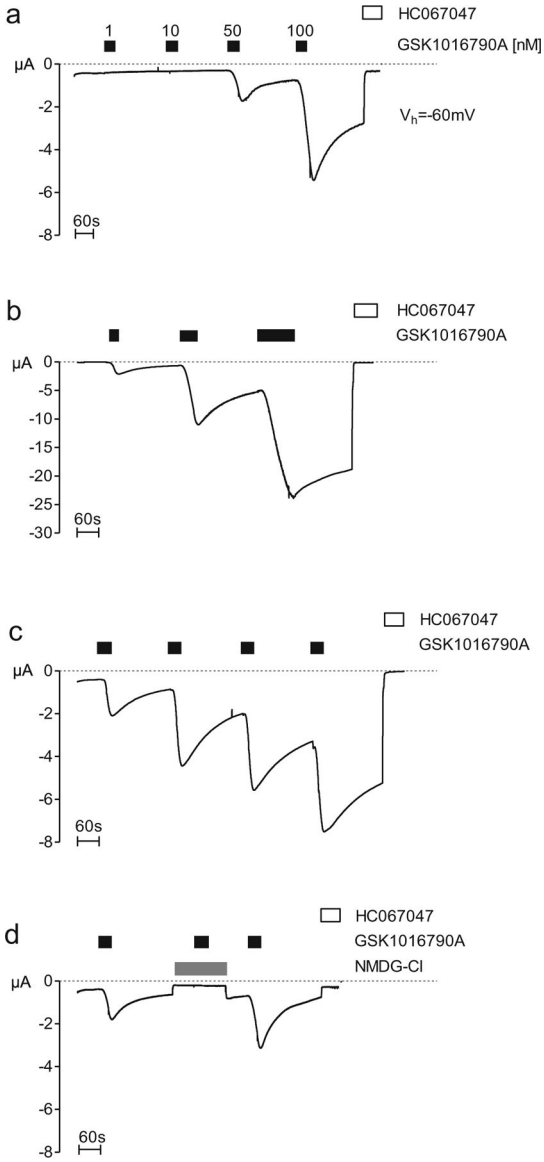

Fig. 1.

Functional evidence for TRPV4 expression in the oocyte expression system. Oocytes were injected with 0.5 ng of cRNA coding for TRPV4 and incubated for 2 days in ND96 solution. GSK1016790A-mediated whole-cell currents were measured using the two-electrode voltage-clamp technique. GSK1016790A and the TRPV4 inhibitor HC067047 were present in the bath solution, as indicated by *closed* and *open bars*, respectively. **a–d** Representative whole-cell current traces of oocytes expressing TRPV4 recorded in Ca^{2+}-free solution. **a** Concentration-dependent effect of GSK1016790A on TRPV4. Concentrations ranging from 1 to 100 nM were used. **b** Time-dependent effect of GSK1016790A on TRPV4. Application times ranging from 30 s to 2 min. **c** Repeated application (four times) of GSK1016790A (50 nM). **d** The period of NMDG-Cl application is indicated by the *grey bar*

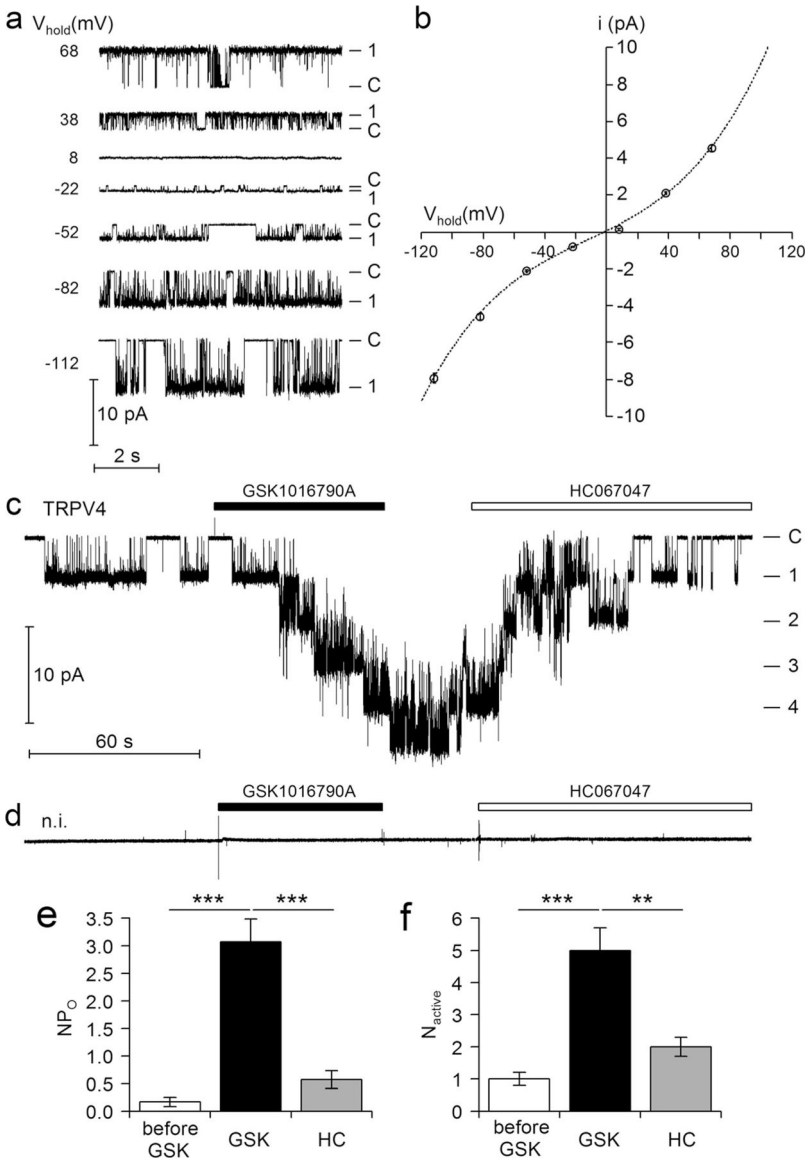

Fig. 2.

Single-channel properties of TRPV4 and its activation by GSK1016790A **a** Representative single-channel current traces at different holding potentials (V_{hold}) from an outside-out patch of an oocyte expressing TRPV4. **b** Average single-channel *I-V* plot calculated from recordings (11 patches from three batches of oocytes) similar to those shown in **a.** Binned current amplitude histograms (not shown for clarity) were used to determine the single-channel current amplitude (*i*) at each holding potential. The *dashed line* represents a polynomial fit of the data. **c, d** Representative single-channel current recordings obtained at V_{hold}=−82 mV from outside-out patches of an oocyte expressing TRPV4 and of a non-injected control oocyte (*n.i.*), respectively. GSK1016790A (50 nM) and HC067047 (100 nM) were present in the bath solution as indicated by the *bars above the traces*. Single-channel traces from experiments as shown in c were further analysed to determine NP_O (**e**) and the number of active channel channels in a patch (N_{active}; **f**) before application

of GSK1016790A (*before GSK*, n= 10), after application GSK1016790A (*GSK*, n=10) and in the presence of HC067047 (*HC*, n=8). **p<0.01; ***p<0.001 unpaired t test

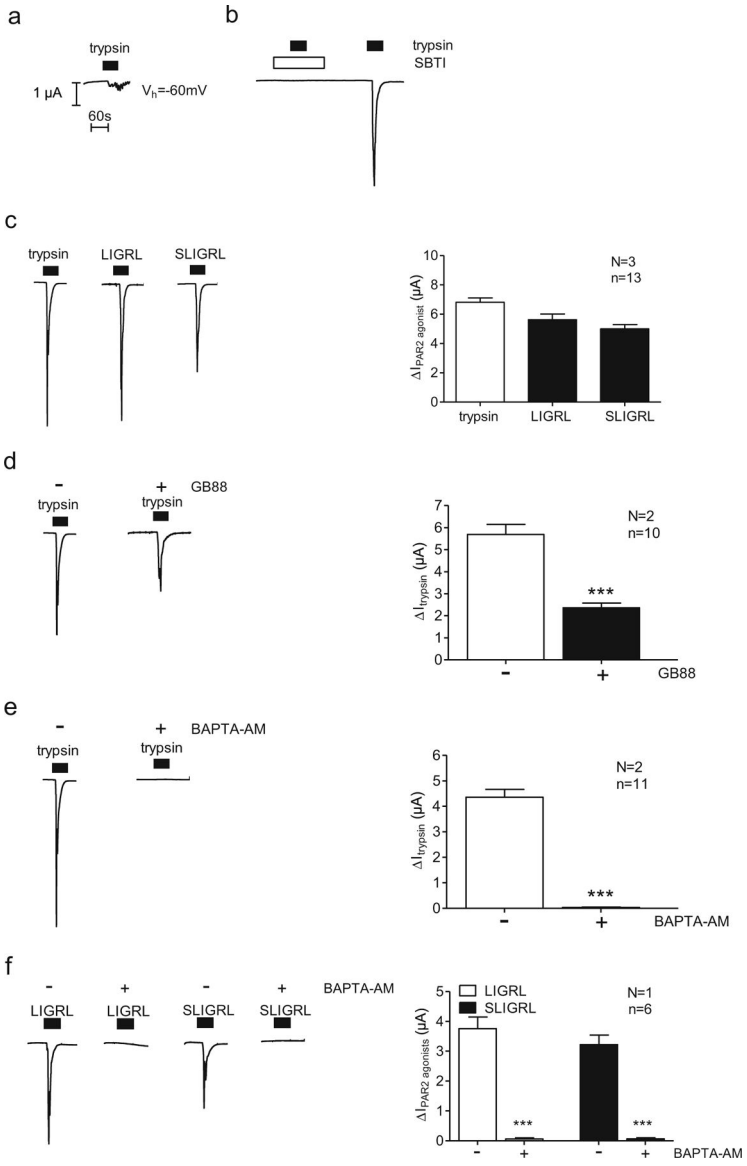

Fig. 3.

Functional evidence for PAR_2 expression in the oocyte expression„ system. Oocytes were injected with 10 ng of cRNA coding for PAR_2 and incubated for 2 days in ND96 solution, or non-injected oocytes were used. The whole-cell currents caused by trypsin or the synthetic activating peptides of PAR_2 (2-furoyl-LIGRL-NH_2 or SLIGRL-NH_2) were measured using the two-electrode voltage-clamp technique. **a–e** Representative whole-cell current traces of a non-injected oocyte (**a**) or oocytes expressing PAR_2 (**b, e**) recorded in Ca^{2+}-free solution. Trypsin (8 nM), LIGRL (10 μM), SLIGRL (100 μM) or the trypsin inhibitor SBTI (0.08 μM) was present in the bath solution, as indicated by *closed* and *open bars*, respectively. *Columns* represent mean $\Delta I_{PAR2\ agonist}$ values from similar experiments. **d** Representative whole-cell current traces of oocytes expressing PAR_2 incubated for 15 min in Ca^{2+}-free solution in the absence (−) or presence (+) of GB88 (10 μM). *Columns* represent mean $\Delta I_{trypsin}$ values from similar experiments. **e, f** Representative whole-cell current traces of

oocytes expressing PAR_2 incubated for 3 h in Ca^{2+}-free solution in the absence (−) or presence (+) of BAPTA-AM (100 μM). *Columns* represent mean $\Delta I_{trypsin}$ or $\Delta I_{PAR2\ agonist}$ values from similar experiments. *n* indicates number of individual oocytes measured. *N* indicates the number of batches of oocytes. ***$p<0.001$, unpaired *t* test

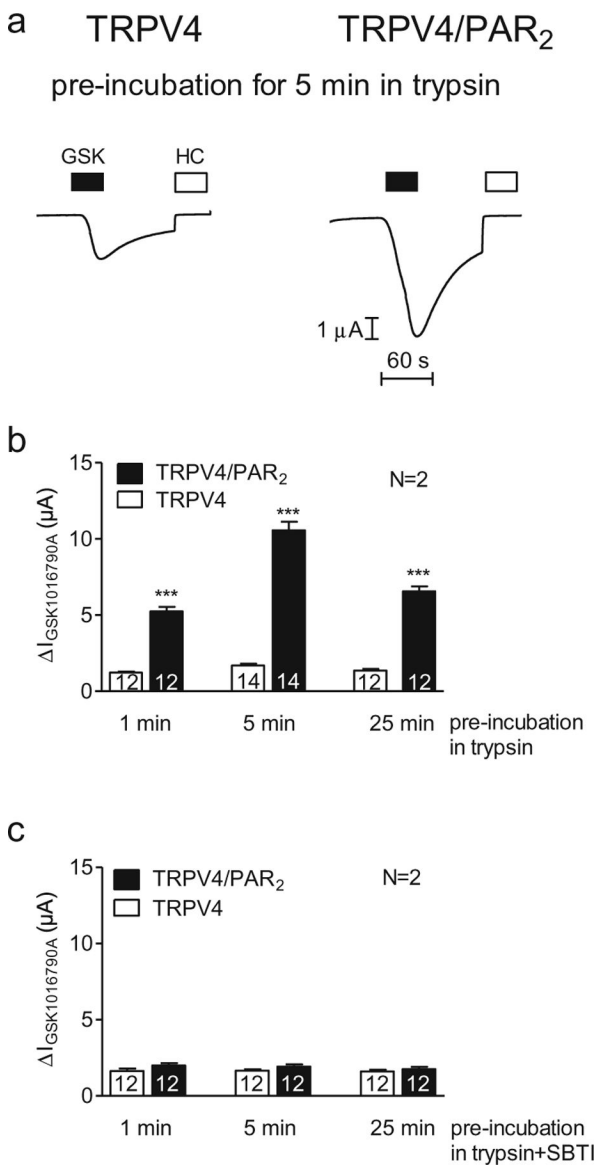

Fig. 4.

Proteolytic activation of PAR_2 by trypsin sensitises TRPV4. Oocytes were injected with cRNA coding for TRPV4 0.5 ng or TRPV4 0.5 ng/PAR_2 10 ng and incubated for 2 days in ND96 solution. GSK1016790A-mediated whole-cell currents were measured using the two-electrode voltage-clamp technique. Activation of TRPV4 by GSK1016790A (50 nM) after incubation in Ca^{2+}-free solution in the presence of trypsin (8 nM) for different time points (1, 5 and 25 min). **a** Representative whole-cell current traces of oocytes expressing TRPV4 (*left side*) or TRPV4/PAR_2 (*right side*) recorded in Ca^{2+}-free solution. Activation and inhibition of TRPV4 by GSK1016790A (*GSK*, *closed bars*; 50 nM) and HC067047 (*HC*, *open bars*; 100 nM), respectively, after pre-incubation for 5 min in Ca^{2+}-free solution in the presence of trypsin (8 nM). **b, c** *Columns* represent mean $\Delta I_{GSK1016790A}$ values of oocytes pre-incubated in trypsin (**b**) or trypsin in the presence of the trypsin inhibitor SBTI

(0.08 μM; **c**). *N* indicates the number of batches of oocytes. *Numbers inside the columns* indicate the number of individual oocytes measured. ***$p<0.001$, unpaired *t* test

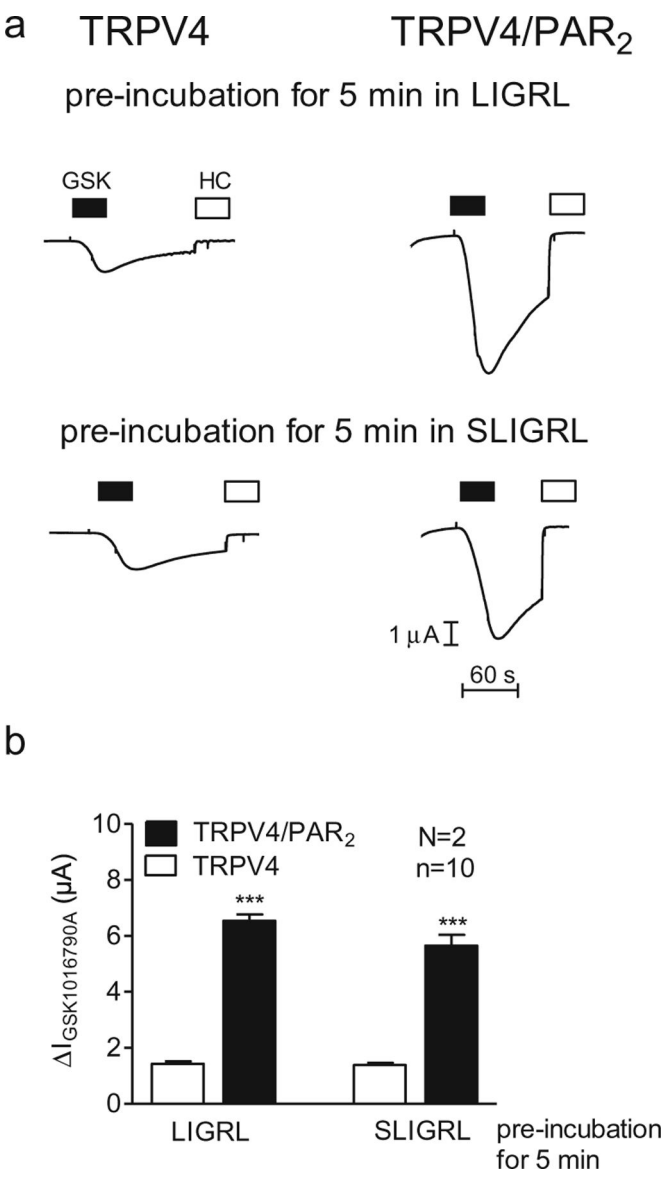

Fig. 5.

Activation of PAR_2 by PAR_2 agonists (2-furoyl-LIGRL-NH_2 or SLIGRL-NH_2) also sensitises TRPV4. Oocytes were injected with cRNA coding for TRPV4 0.5 ng or TRPV4 0.5 ng/PAR_2 10 ng and incubated for 2 days in ND96 solution. GSK1016790A-mediated whole-cell currents were measured using the two-electrode voltage-clamp technique. Activation of TRPV4 by GSK1016790A (50 nM) after incubation for 5 min in Ca^{2+}-free solution in the presence of LIGRL (10 µM) or SLIGRL (100 µM). **a** Representative whole-cell current traces of oocytes expressing TRPV4 (*left side*) or TRPV4/PAR_2 (*right side*) recorded in Ca^{2+}-free solution. Activation or inhibition of TRPV4 by GSK1016790A (*GSK*, *closed bars*; 50 nM) or HC067047 (*HC*, *open bars*; 100 nM), respectively. **b** *Columns* represent mean $\Delta I_{GSK1016790A}$ values. *n* indicates number of individual oocytes measured. *N* indicates the number of batches of oocytes. ***$p<0.001$, unpaired *t* test

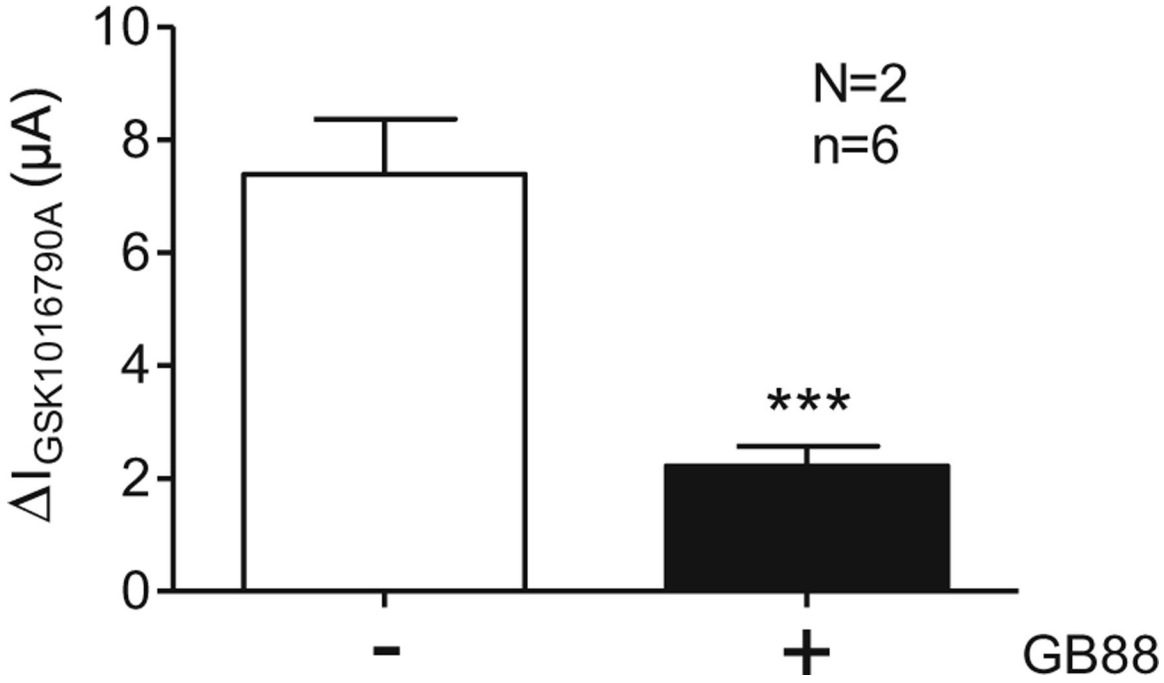

Fig. 6.

Inhibition of PAR_2 by GB88 causes a reduction of TRPV4 sensitisation. Oocytes were injected with cRNA coding for TRPV4 0.5 ng/PAR_2 10 ng and incubated for 2 days in ND96 solution. GSK1016790A-mediated whole-cell currents were measured using the two-electrode voltage-clamp technique. Oocytes were pre-incubated for 15 min in Ca^{2+}-free solution in the absence (−) or in the presence (+) of GB88 (10 μM). After a subsequent pre-incubation for 5 min in trypsin, TRPV4 was activated by GSK1016790A (50 nM). *Columns* represent mean $\Delta I_{GSK1016790A}$ values. *n* indicates number of individual oocytes measured. *N* indicates the number of batches of oocytes. ***$p<0.001$, unpaired *t* test

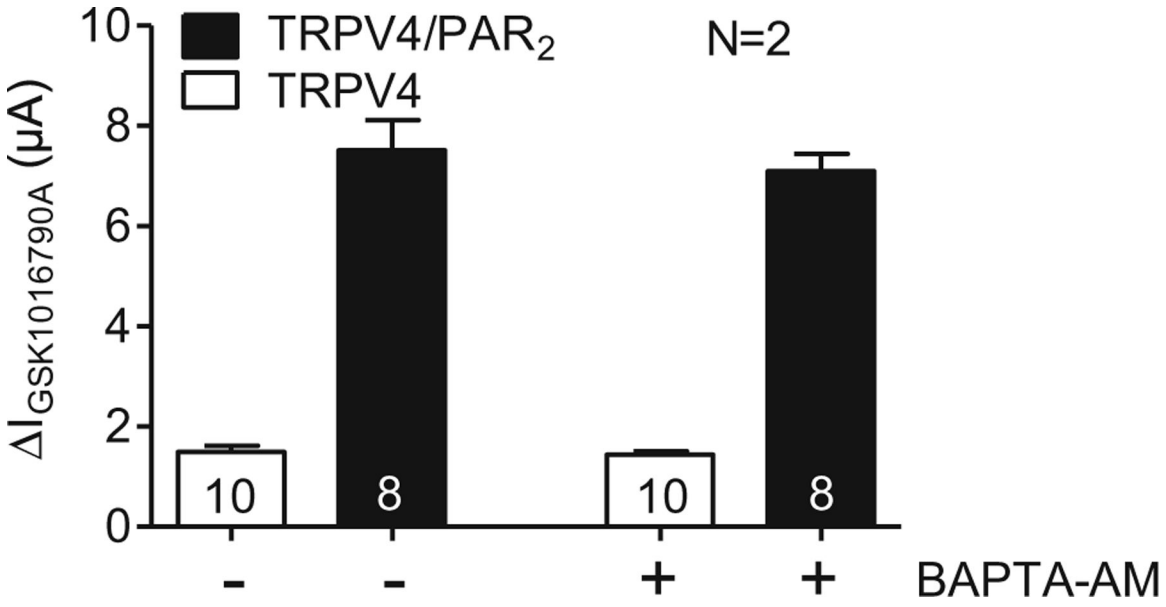

Fig. 7.

PAR_2-mediated sensitisation of TRPV4 is not affected by BAPTA-AM. Oocytes were injected with cRNA coding for TRPV4 0.5 ng or TRPV4 0.5 ng/PAR_2 10 ng and incubated for 2 days in ND96 solution. GSK1016790A-mediated whole-cell currents were measured using the two-electrode voltage-clamp technique. Oocytes were pre-incubated for 3h in Ca^{2+}-free solution in the absence (−) or in the presence (+) of BAPTA-AM (100 μM). After a subsequent pre-incubation for 5 min in trypsin (8 nM), TRPV4 was activated by GSK1016790A (50 nM). *Columns* represent mean $\Delta I_{GSK1016790A}$ values. *N* indicates the number of batches of oocytes. *Numbers inside the columns* indicate the number of individual oocytes measured

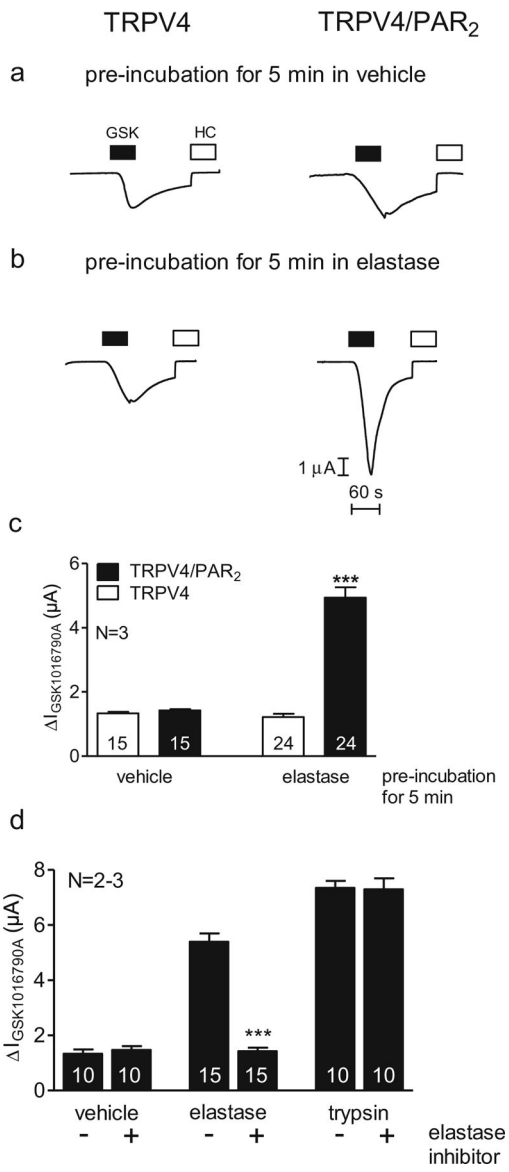

Fig. 8.

Proteolytic activation of PAR_2 by the biased agonist human neutrophil elastase sensitises TRPV4. Oocytes were injected with cRNA coding for TRPV4 0.5 ng or TRPV4 0.5 ng/PAR_2 10 ng and incubated for 2 days in ND96 solution. GSK1016790A-mediated whole-cell currents were measured using the two-electrode voltage-clamp technique. **a, b** Representative whole-cell current traces of oocytes expressing TRPV4 (*left side*) or TRPV4/PAR_2 (*right side*) recorded in Ca^{2+}-free solution. Activation and inhibition of TRPV4 by GSK1016790A (*GSK*, *closed bars*; 50 nM) and HC067047 (*HC*, *open bars*; 100 nM), respectively, after pre-incubation for 5 min in only Ca^{2+}-free solution (vehicle; **a**) or in Ca^{2+}-free solution in the presence of elastase (3 µM; **b**). **c** *Columns* represent mean $\Delta I_{GSK1016790A}$ values. **d** *Columns* represent mean $\Delta I_{GSK1016790A}$ of oocytes expressing TRPV4/PAR_2. *N* indicates the number of batches of oocytes. *Numbers inside the columns* indicate the number of individual oocytes measured. ***$p<0.001$, unpaired *t* test

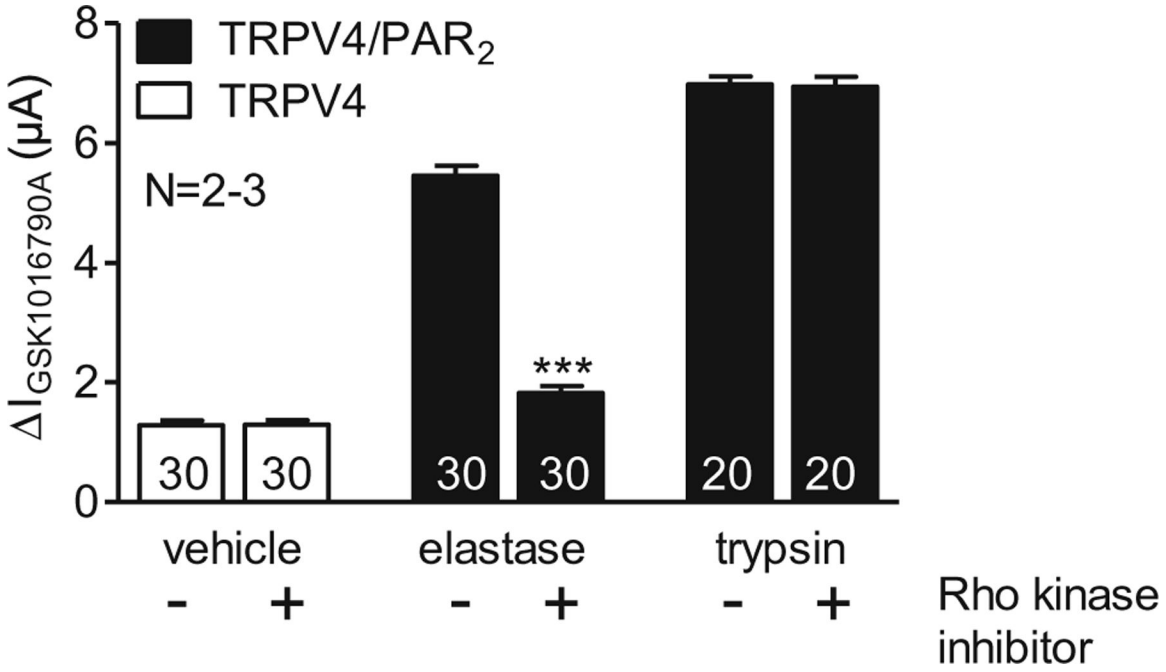

Fig. 9.

Rho-kinase inhibitor Y27362 inhibits PAR_2-mediated sensitisation of TRPV4 by neutrophil elastase Oocytes were injected with cRNA coding for TRPV4 0.5 ng or TRPV4 0.5 ng/ PAR_2 10 ng and incubated for 2 days in ND96 solution. GSK1016790A-mediated whole-cell currents were measured using the two-electrode voltage-clamp technique. Oocytes expressing TRPV4 (*white bars*) were pre-incubated for 15 min in Ca^{2+}-free solution in the absence (−) or in the presence (+) of Rho-kinase inhibitor Y27362 (10 μM). Oocytes expressing TRPV4/PAR_2 (*black bars*) were pre-incubated for 15 min in elastase (3 μM) or trypsin (8 nM) in the absence (−) or in the presence (+) of Rho-kinase inhibitor (10 μM). *Columns* represent mean $\Delta I_{GSK1016790A}$ values. *N* indicates the number of batches of oocytes. *Numbers inside the columns* indicate the number of individual oocytes measured. ***$p<0.001$, unpaired *t* test