Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P_2-mediated inhibition

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Tissue injury generates endogenous factors that heighten our sense of pain by increasing the response of sensory nerve endings to noxious stimuli[1,2]. Bradykinin and nerve growth factor (NGF) are two such pro-algesic agents that activate G-protein-coupled (BK_2) and tyrosine kinase (TrkA) receptors, respectively, to stimulate phospholipase C (PLC) signalling pathways in primary afferent neurons[3,4]. How these actions produce sensitization to physical or chemical stimuli has not been elucidated at the molecular level. Here, we show that bradykinin- or NGF-mediated potentiation of thermal sensitivity *in vivo* requires expression of VR1, a heat-activated ion channel on sensory neurons. Diminution of plasma membrane phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) levels through antibody sequestration or PLC-mediated hydrolysis mimics the potentiating effects of bradykinin or NGF at the cellular level. Moreover, recruitment of PLC-γ to TrkA is essential for NGF-mediated potentiation of channel activity, and biochemical studies suggest that VR1 associates with this complex. These studies delineate a biochemical mechanism through which bradykinin and NGF produce hypersensitivity and might explain how the activation of PLC signalling systems regulates other members of the TRP channel family.

Nociceptors are specialized sensory neurons that detect chemical, physical or mechanical stimuli that could cause tissue damage. Inflammation increases the levels of extracellular protons, neurotransmitters and growth factors within the local tissue environment, thereby decreasing activation thresholds and enhancing the excitability of the nociceptor[2,5]. Included among these agents are the nonapeptide bradykinin and the neurotrophin NGF[2,6–8]. Sensitization by bradykinin or NGF might involve long-term changes in gene expression[3,9,10], but the ability of these factors to promote nociceptor sensitization within several minutes suggests that post-translational mechanisms are also involved. The downstream effector molecules that mediate sensitization by bradykinin or NGF have not been fully characterized[3].

One candidate target on nociceptors is VR1, a cation channel that is activated by pungent vanilloid compounds (such as capsaicin), extracellular protons or noxious heat[11]. This ability to integrate thermal and chemical stimuli makes VR1 potentially well suited to modulate sensitivity of the nociceptor after tissue injury[12]. Indeed, VR1-deficient mice do not develop thermal hyperalgesia in response to inflammation[13,14]. Because injury generates an array of chemical mediators, we asked whether bradykinin and NGF, specifically, contribute to VR1-dependent thermal hypersensitivity. We injected wild-type and VR1-deficient mice with bradykinin or NGF and measured paw withdrawal latencies from a radiant heat source before and after treatment. Each agent produced substantial sensitization in wild-type animals but not in VR1-null mice (Fig. 1), demonstrating that VR1 is essential for the development of bradykinin- or NGF-induced thermal hypersensitivity *in vivo*.

Whereas extracellular protons interact directly with VR1 to potentiate its sensitivity to capsaicin or heat[12,15], NGF or bradykinin might modulate VR1 by binding to their own receptors on sensory neurons and activating second messenger signalling cascades. Indeed, VR1 belongs to the TRP channel family, some members of which are activated downstream of PLC-coupled receptors through as-yet undefined mechanisms[16]. To address this possibility, we expressed VR1 and BK_2 receptors together in human embryonic kidney (HEK293) cells and examined their sensitivity to VR1 agonists before and after exposure to bradykinin. Moderately acidic solutions (pH 6.4) evoked small but discernible inward currents at negative holding potentials. Subsequent treatment with bradykinin (20 nM) dramatically increased these responses ~25-fold (Fig. 2a). Even at physiological pH, bradykinin produced a slight increase in holding current that was attributable to VR1, based on characteristic outward rectification and inhibition by the vanilloid receptor antagonist capsazepine (see Supplementary Information). At a low dose of capsaicin (10 nM), bradykinin also produced a substantial (approximately fivefold), prolonged potentiation of evoked currents (Fig. 2b). In all cases, modulation was observed only in cells expressing both VR1 and BK_2 receptors.

To determine whether potentiation involves a change in channel gating, we analysed the kinetic properties of capsaicin-evoked

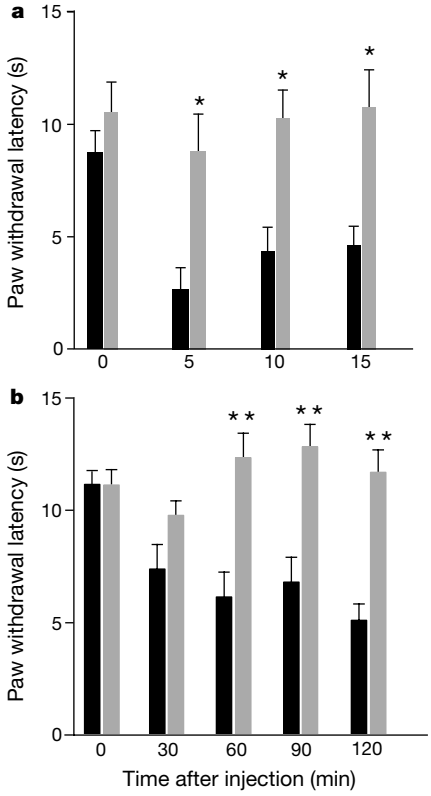

Figure 1 VR1 is essential for the development of bradykinin- or NGF-evoked thermal hypersensitivity *in vivo*. **a**, Wild-type (black) or $VR1^{-/-}$ (grey) mice were injected intraplantarly with bradykinin and the response latency to radiant heating of the hind paw was measured at various times after injection (zero time point shows pre-injection response latency). *: $P < 0.05$ for $VR1^{+/+}$ versus $VR1^{-/-}$; $P = 0.02$ for entire time course; $n = 7$ mice per genotype. **b**, NGF-evoked thermal hypersensitivity was measured as above. **: $P < 0.02$ for $VR1^{+/+}$ versus $VR1^{-/-}$; $P = 0.01$ for the entire time course; $n = 7$ mice per genotype. NGF was administered subcutaneously and delivery to the receptive field (that is, the paw) might account for the slower onset of hyperalgesia relative to bradykinin. Neither group showed significant change in response latencies following saline injection (not shown).

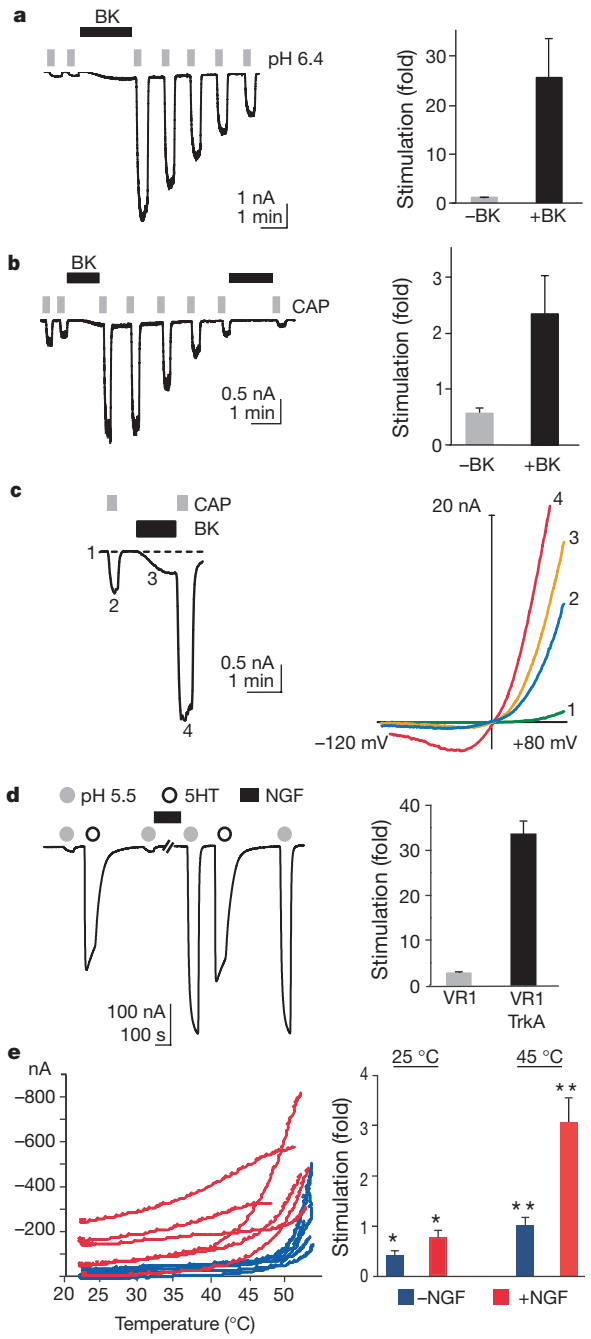

Figure 2 Bradykinin (BK) and nerve growth factor (NGF) sensitize VR1 in heterologous expression systems. **a**, Left: BK (20 nM) potentiation of proton-evoked (pH 6.4) responses in a voltage-clamped VR1-expressing HEK293 cell. Right: normalized proton response before (grey) and after (black) BK application ($n = 8$, $P < 0.05$). **b**, Left: BK potentiation of capsaicin-evoked (CAP; 10 nM) responses in a voltage-clamped VR1-expressing HEK293 cell. Right: capsaicin response normalized to a previous application before (grey) and after (black) BK treatment ($n = 16$, $P < 0.05$). **c**, Current–voltage relationships of VR1 responses to capsaicin (10 nM) before and after BK application reveal a change in gating. Numbers indicate points at which voltage-ramp traces (right) were obtained from the current trace (left). **d**, Left: NGF sensitizes proton-evoked currents in oocytes expressing VR1, TrkA, p75 and $5HT_3R$-A. Break indicates 10 min NGF (0.75 nM) application. Protons (pH 5.5) and serotonin (5HT, 10 μM) were perfused for 30 s. Right: averaged potentiation by NGF in these cells (black) versus oocytes expressing VR1 alone (grey; $n = 10$, $P < 0.001$). **e**, Heat-evoked currents in oocytes co-expressing TrkA. Cells were incubated with NGF (red, 0.75 nM) or vehicle; (blue; ND-96 buffer, pH 7.5) for 10 min at room temperature before analysis ($n = 14$–16; *, $P < 0.05$; **, $P < 0.001$). Water-injected oocytes did not respond to heat (not shown). Averaged values (right) were normalized to current responses of vehicle-treated cells at 45 °C.

currents before and after bradykinin exposure. In the absence of any stimulus, VR1 displayed slight basal channel activity characterized by strong outward rectification; the inward current at negative holding potential was negligible (Fig. 2c, curve 1). Application of capsaicin produced a greater proportional increase in currents at negative membrane potentials, resulting in weaker outward rectification (Fig. 2c, curve 2). Upon activation of BK_2 receptors, capsazepine-inhibitable basal currents grew slowly over 2–3 min and rectification gradually weakened (Fig. 2c, curve 3). A second challenge with capsaicin further weakened the outward rectification, preferentially augmenting the inward component (Fig. 2c, curve 4). A similar phenomenon was observed when protons were used as agonist (not shown). These observations suggest that potentiation of VR1 by bradykinin primarily involves an alteration in channel gating.

Previous studies have shown that exposing cultured dorsal root ganglion (DRG) neurons to NGF for 10 min produces acute sensitization of capsaicin-evoked responses[17]. Similarly, we found that NGF produced robust (~30-fold) increases in proton-evoked

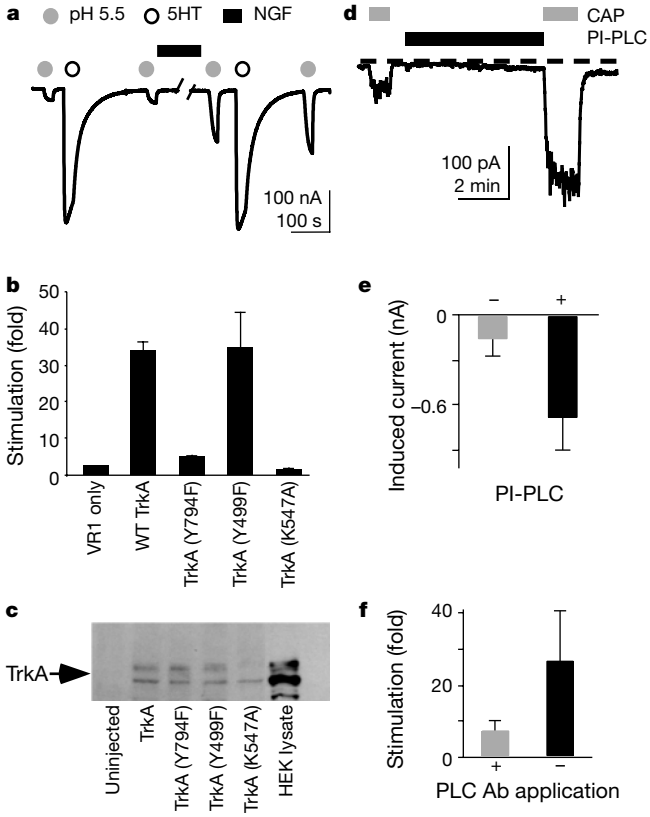

Figure 3 Phospholipase C is involved in nerve growth factor (NGF) modulation of VR1 function. **a**, Proton-evoked (pH 5.5) responses in a voltage-clamped oocyte expressing VR1, TrkA (Y794F), p75 and $5HT_3R$-A. Analysis was performed as described in Fig. 2 ($P < 0.001$ versus cells expressing wild-type TrkA; $n = 10$). Break indicates 10 min NGF (0.75 nM) application. Protons (pH 5.5) and serotonin (5HT) (10 μM) were perfused for 30 s. **b**, Histogram of the average potentiation of proton-evoked responses in oocytes co-expressing VR1 and wild-type or mutant TrkA receptors. **c**, Western blot of oocyte extracts indicates similar levels of wild type and mutant TrkA receptor expression. Lysate from TrkA-transfected HEK293 cells provides a control for TrkA immunoreactivity. **d**, **e**, Capsaicin-evoked (20 nM) currents before (grey) and after (black) application of inositide-phospholipid-specific phospholipase C (PI-PLC; 0.5 U ml^{-1}) to inside-out membrane patches excised from HEK293 cells expressing VR1 (–60 mV holding potential; $n = 12$, $P < 0.01$). **f**, PI-PLC potentiation (black) was reduced on pretreatment of PI-PLC with the anti-PLC antibody 72-24 (PLCAb; grey). Current was normalized to first capsaicin application ($n = 7$, $P < 0.05$).

(pH 5.5) currents in oocytes expressing both TrkA and VR1 (Fig. 2d). Potentiation was specific for VR1 because we found no change in the activity of co-expressed $5HT_3$ serotonin-gated channels. Moreover, NGF had no effect unless TrkA was present (Fig. 2d). We also compared temperature response profiles for NGF-treated and untreated cells, and found that NGF produced a substantial decrease in the thermal threshold for VR1 activation, such that significant currents were observed even at room temperature (Fig. 2e). This was accompanied by an increase in the magnitude of responses at temperatures both below and above the threshold normally required for VR1 activation (~43 °C). These effects, as well as those described above for bradykinin, are reminiscent of the signature changes in nociceptor excitability produced by inflammation, including development of ongoing activity and sensitization to thermal stimuli[10]. Although the neurotrophin receptor p75 was usually expressed together with TrkA in these experiments, no difference was observed in its absence (not shown), which is consistent with recent evidence that p75 is not required for NGF-evoked thermal hypersensitivity *in vivo*[18].

PLC stimulation is common to both NGF and bradykinin receptor activation, and we therefore asked whether this is an obligate step in the VR1 potentiation process. A TrkA mutant (Y794F) that specifically abrogates recruitment of PLC-γ to the receptor complex[19] showed greatly diminished NGF-mediated potentiation of proton-evoked currents (14 ± 7% of the wild-type receptor), even though western blot analysis showed that mutant and wild-type receptors were expressed at comparable levels (Fig. 3a–c). By contrast, another TrkA mutant (Y499F) that disrupts coupling to the mitogen-activated-protein-kinase pathway[19] was fully capable of potentiating VR1-evoked responses (Fig. 3a–c). These results demonstrate that PLC activation is the predominant pathway involved in VR1 potentiation by NGF. To determine whether PLC activity alone can potentiate VR1 responses, we applied a recombinant inositide-phospholipid-specific PLC (PI-PLC) directly to patches excised from VR1-expressing HEK293 cells. PI-PLC enhanced both basal channel activity and capsaicin-evoked responses, and these effects were significantly reduced by a neutralizing monoclonal antibody to the enzyme (Fig. 3d–f).

PLC catalyses the hydrolysis of membrane PtdIns(4,5)P_2 to yield inositol triphosphate and diacylglycerol, one consequence of which is activation of protein kinase C (PKC). Pharmacological studies suggest that bradykinin potentiates heat-evoked currents in sensory

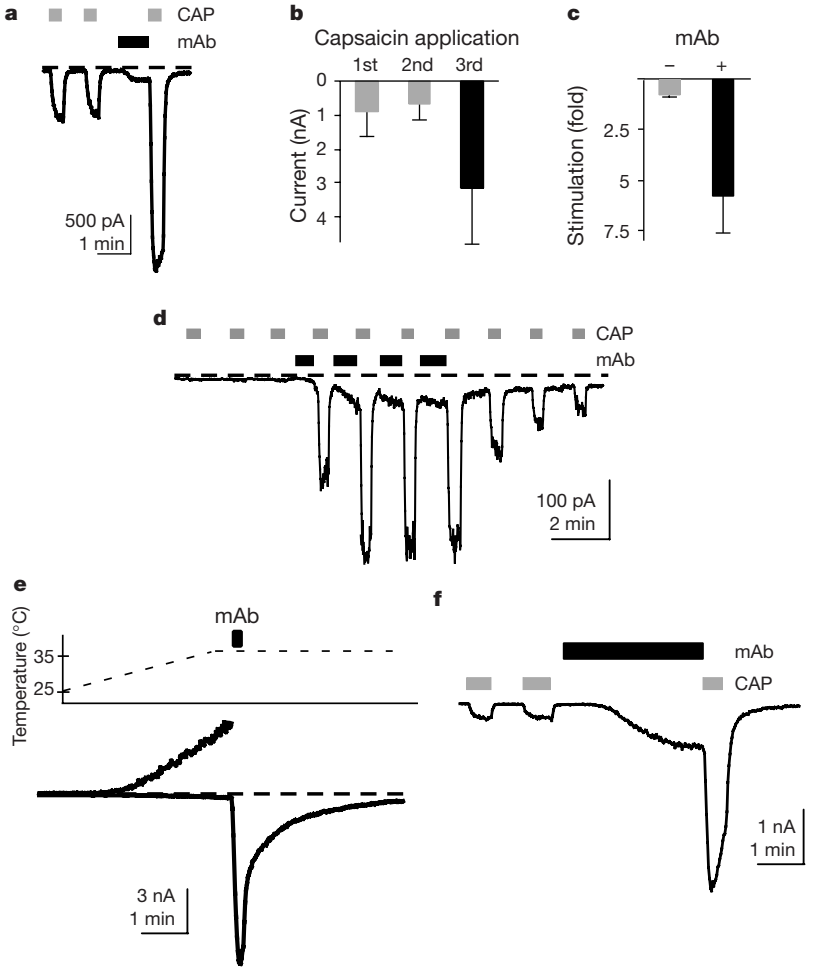

Figure 4 Phosphatidyl-4,5-inositol bisphosphate (PtdIns(4,5)P_2) antibody application mimics modulation of VR1 by bradykinin (BK) or nerve growth factor (NGF). **a**, Application of PtdIns(4,5)P_2 monoclonal antibody (mAb) to an inside-out membrane patch excised from VR1-expressing oocytes induced basal current and potentiated capsaicin (CAP, 20 nM)-evoked currents. **b**, Averaged response to capsaicin challenge in the absence (1st and 2nd; grey) and presence (3rd; black) of mAb. **c**, Current responses induced by capsaicin (normalized to previous application) showed more than sixfold potentiation by mAb ($n = 12$, $P < 0.001$). **d**, Modulation by mAb was also observed in membrane patches excised from VR1-expressing HEK293 cells. **e**, Heat application to an excised membrane patch from a VR1-expressing HEK293 cell elicited a strongly rectifying basal current below 37 °C (upward deflection shows outward current at +70 mV holding potential before mAb application). Brief application of mAb then provoked a large inward current (–60 mV holding potential) at 37 °C. **f**, Potentiation of capsaicin-evoked (20 nM) responses by mAb was also observed in membrane patches excised from rat DRG neurons.

neurons through activation of PKC[20], and that PKC potentiates capsaicin-evoked responses in VR1-expressing oocytes[21]. However, we found that neither staurosporine nor Ro-31-8425, two PKC inhibitors, blocked NGF-elicited potentiation of VR1 in oocytes (not shown). In addition, attempts to mimic potentiation with the PKC activator phorbol 12-myristate 13-acetate (PMA, also known as TPA) were confounded by our observation that this agent antagonizes binding of [^{3}H]-resiniferatoxin (a high-affinity capsaicin receptor agonist) to VR1, suggesting that PMA interacts directly with the channel (see Supplementary Information). Importantly, we also found that PI-PLC potentiated VR1 currents in the absence of ATP.

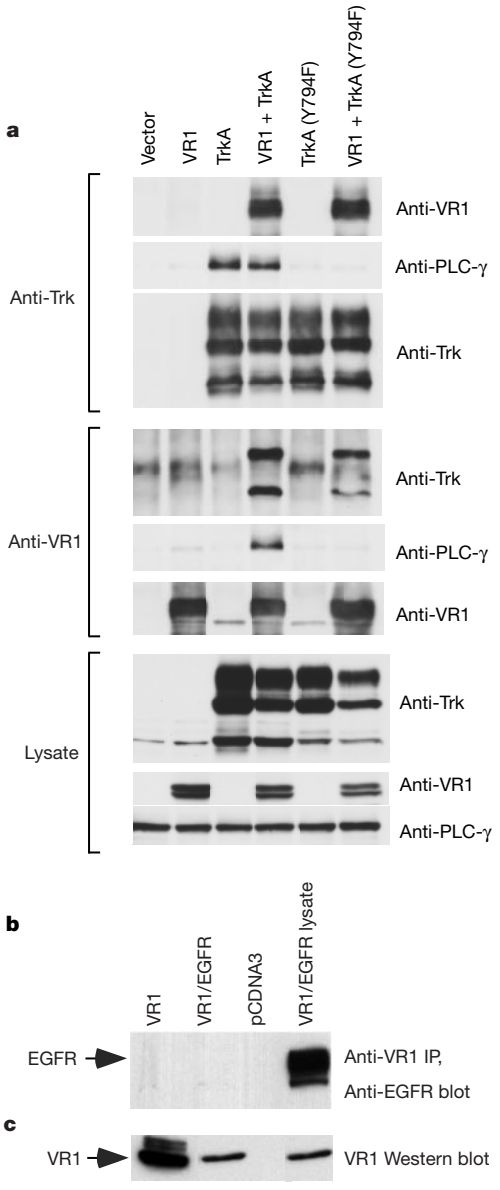

Figure 5 VR1, TrkA and phospholipase Cγ (PLC-γ) form a signalling complex. **a**, HEK293 cells were transfected with the indicated cDNAs (top). Immunoprecipitates were formed with the antibodies indicated on the right and visualized on western blots with the antibodies indicated on the left. Total lysates were also analysed to demonstrate equivalent protein expression among samples. TrkA is typically produced as three differently glycosylated isoforms migrating as species with relative molecular masses of 140,000, 110,000 and 80,000 (M_r 140K, 110K and 80K, respectively). **b**, VR1 does not form a complex with the epidermal growth factor receptor (EGFR). HEK293 cells were transfected with indicated cDNAs and immune complexes formed using VR1 antiserum. Blots were probed with EGFR antibody. The rightmost lane contains lysate from cells producing both VR1 and EGFR. **c**, VR1 expression in total cell lysates.

Taken together, these results suggest that modulation of VR1 involves both PKC-dependent and PKC-independent mechanisms, with the latter possibly occurring as a direct consequence of PtdIns(4,5)P_2 hydrolysis. A number of ion channels are regulated in this manner, as exemplified by PtdIns(4,5)P_2 inhibition of cyclic nucleotide-gated channels[22] or PtdIns(4,5)P_2 potentiation of G-protein-gated inwardly rectifying potassium channels[23,24]. Anti-PtdIns(4,5)P_2 monoclonal antibodies sequester membrane PtdIns(4,5)P_2 and thus shift the sensitivity of these channels to cyclic GMP or $G_{\beta\gamma}$, respectively. When we applied this antibody to the cytoplasmic side of excised inside-out patches from VR1-expressing oocytes, we observed a gradual stimulation of basal channel activity (Fig. 4a–c) that displayed characteristic outward rectification. PtdIns(4,5)P_2 antibody also potentiated capsaicin-evoked currents, which normally display tachyphylaxis (a gradual reduction of induced current upon repetitive capsaicin application). Additionally, when the pipette solution was made more acidic (pH 6.4), PtdIns(4,5)P_2 antibody induced large inward currents (not shown). A similar induction was observed at neutral pH in cells expressing the VR1 mutants E600Q or E600K (not shown), which, under normal physiological conditions, behave like proton-potentiated wild-type channels[15]. PtdIns(4,5)P_2 antibody did not evoke responses in membrane patches from control oocytes (not shown). Taken together, these results demonstrate that PtdIns(4,5)P_2-antibody-activated currents are carried by VR1.

Cell types differ in their membrane lipid composition and we asked whether PtdIns(4,5)P_2 sequestration would also sensitize VR1 channels in mammalian cells, including sensory neurons. Very small inward currents were observed when 20 nM capsaicin was applied to inside-out membrane patches excised from VR1-transfected HEK293 cells. Application of PtdIns(4,5)P_2 antibody enhanced basal and capsaicin-induced currents. Both of these currents were suppressed by capsazepine (see Supplementary Information) and antibody effects were partially reversed on washout (Fig. 4d). This action was observed only when antibody was applied to the cytoplasmic side of the membrane (where PtdIns(4,5)P_2 resides) and a monoclonal antibody against progesterone had no effect (not shown). PtdIns(4,5)P_2 antibody also shifted the heat sensitivity of VR1 to a lower temperature range. When excised patches from these cells were exposed to a temperature ramp plateauing at 37 °C, a small VR1-mediated basal current developed. Brief application of antibody to this membrane patch then triggered a large inward current, indicating that the thermal sensitivity of VR1 can be modulated by PtdIns(4,5)P_2 in a membrane-delimited fashion (Fig. 4e). PtdIns(4,5)P_2 antibody also stimulated basal currents and sensitized capsaicin-evoked responses in rat DRG neurons (Fig. 4f). These results suggest that endogenous PtdIns(4,5)P_2 inhibits VR1 and that repression can be alleviated by agents that activate PLC.

Mammalian TRPC3 channels associate with TrkB receptors in the brain and these channels can be stimulated by brain-derived neurotrophic factor through a TrkB/PLC-dependent signalling pathway[25]. We therefore asked whether VR1 and TrkA associate to form an analogous signalling complex. Immunoprecipitates prepared from HEK293 cells co-expressing VR1 and TrkA contained both proteins, irrespective of whether VR1 or TrkA antiserum was used to form the precipitate (Fig. 5a). Moreover, endogenous PLC-γ was detected in these samples, demonstrating that VR1, TrkA and PLC-γ can associate to form a ternary complex. Significantly less PLC-γ was recovered in immunoprecipitates from cells producing TrkA (Y794F) mutant receptors (Fig. 5a), which is consistent with our electrophysiology results showing reduced NGF potentiation of VR1 currents in oocytes producing this construct. In addition, VR1 associated with a catalytically inactive TrkA (K547A) mutant (see Supplementary Information), demonstrating that receptor kinase

activity is not a prerequisite for interaction. Although we have not shown that TrkA and VR1 associate in sensory neurons, the interaction that we observe in HEK293 cells is specific because the epidermal growth factor (EGF) receptor, a closely related receptor tyrosine kinase, does not immunoprecipitate with VR1 when expressed in these cells (Fig. 5b, c).

A subset of vertebrate and invertebrate TRP channels are activated by neurotransmitter, hormone or growth-factor receptors that couple to PLC signalling pathways. How PLC activation leads to TRP channel opening is still the subject of significant debate, but a number of mechanisms have been proposed that involve calcium store depletion, direct interaction with inositol triphosphate receptors or activation by lipid metabolites produced downstream of PLC[16]. Our data suggest that VR1 can be activated or potentiated at a very early point in the pathway as an immediate consequence of PLC activation and PtdIns(4,5)P_2 hydrolysis. Indeed, application of $G_{\alpha 11}$ to inside-out membrane patches activates *Drosophila* TRPL channels through a PLC-mediated mechanism[16], and application of PtdIns(4,5)P_2 partially inhibits TRPL opening[26]. The ability of exogenously applied anandamide or diacylglycerol to activate VR1 or other TRP channels in a PKC-independent manner[27,28] raises the possibility that these lipid messengers mediate their effects by displacing PtdIns(4,5)P_2 or other membrane lipids from an inhibitory site on the channel complex.

In the *Drosophila* eye, TRP channels exist in a complex with other components of the phototransduction machinery, an arrangement that influences the sensitivity and kinetics of the signalling process[29,30]. Whether a similar signalling scaffold exists in nociceptors remains to be determined. Interestingly, we have found that a variety of PLC-coupled receptors modulate VR1 function in heterologous systems (not shown). Moreover, preliminary experiments suggest that EGF receptor activation also sensitizes VR1, albeit to a lesser extent (approximately fivefold) than that typically observed with NGF (see Supplementary Information). Thus, whereas the formation of a specific protein complex is not a prerequisite for modulation, complex formation might facilitate the process. Activation of different PLC-coupled receptors by individual components of the inflammatory milieu might allow for spatially and quantitatively distinct mechanisms of nociceptor sensitization. □

Methods

Behaviour

Bradykinin (20 μl of a 10 nM solution in 0.9% saline) was injected intraplantarly into one hind paw of wild-type or $VR1^{-/-}$ mice (adult males, ~25 g body weight) and response latencies to a radiant heat source were measured as described[13]. NGF (2.5 S, gift of W. Mobley) was administered subcutaneously (1 mg kg^{-1} in 0.9% saline). Significance was determined using repeated analysis of variance with Fisher's PLSD (protected least significant difference) *post-hoc* analysis.

Oocyte electrophysiology

Defolliculated stage VI *Xenopus laevis* oocytes were injected with 0.1–1.0 ng rat VR1, rat TrkA, rat p75, human EGFR or mouse $5HT_3$R-A *in vitro* transcribed RNA. Oocytes were maintained in modified Barth's saline solution for 4–6 days before analysis. Two-electrode voltage-clamp analysis was performed using a Geneclamp 500 amplifier (Axon Instruments) and MacLab A/D converter (−45 mV holding potential). ND-96 (with Ba^{2+}) recording solution was buffered with 10 mM Na-MES (pH 5.5) or 10 mM Na-HEPES (pH 7.5) and bath chamber perfused at ~1 ml min^{-1}. Temperature ramps were generated as described[12]. For patch-clamp experiments, 2–10 ng of VR1 cRNA were injected into oocytes and responses measured 2–5 weeks after injection. Recombinant rat NGF-β, human EGF (Sigma) and bradykinin (Bachem) were diluted into the perfusate.

Mammalian cell electrophysiology

HEK293 cells were cultured in DMEM/F12 with 10% FBS (fetal bovine serum) and transfected using Lipofectamine, Superfect or Fugene 6 according to manufacturers' protocols. Cells were transfected with 400 ng plasmid DNA encoding rat VR1, with or without 400 ng human BK_2 receptor complementary DNA. To identify transfected cells, an enhanced green fluorescence protein reporter plasmid was also transfected at one-tenth the concentration of receptor cDNAs. Cells were plated onto coverslips 1–3 days before recording and examined 4–7 days after transfection. Dissociated neurons from rat or mouse DRG were prepared as described[13]. Voltage-clamp experiments were performed at −60 mV holding potential with 320 ms voltage ramp from −120 mV to +80 mV at 1 Hz. Data were acquired using pClamp (Axon Instruments) or Pulse-Pulsefit (HEKA GmBH) software. Recordings were filtered at 5 kHz and sampled at 1 kHz. Standard bath solution for patch-clamp experiments contained 10 mM Tris/HCl, 1 mM EGTA, 1 mM $MgCl_2$ and 150 mM CsCl at pH 7.4. The pH 6.4 solution contained 20 mM citric acid and 1 mM $MgCl_2$, titrated with CsOH to pH 6.4 and supplemented with CsCl to make the final Cs^+ concentration 150 mM. For excised patch experiments, standard bath solution was used in both the pipette and perfusion solution. No ATP was added to the solutions. Diameters of patch pipettes were 20–50 μm for oocyte recordings and 12–15 μm for HEK293 cells or neurons. For whole-cell recording, a 0.9× standard bath solution supplemented with 0.24 mM $CaCl_2$, 0.2 mM Na_2GTP and 0.3 mM Na_2ATP was used in the pipette, adjusted with Tris-base to a final pH of 7.3. Pipette resistance in this solution was typically 2–3 MΩ. PI-PLC and its neutralizing antibody were from Molecular Probes, and PtdIns(4,5)P_2 and progesterone antibodies from Perseptive Biosystem. Titres of PtdIns(4,5)P_2 antibody used in individual experiments were 1:200 (Fig. 4a–c, e), 1:2,000 (Fig. 4d) and 1:500 (Fig. 4f). A perfusion stage with heating resistors was used to generate a heat ramp plateauing at 37 °C, and the bath temperature was monitored with probes (Warner Instruments).

Biochemistry

HEK293 cells were maintained in DMEM containing 10% FBS. Cells were plated at 70–80% confluency and subjected to calcium phosphate or lipofectamine transfection with approximately equal amounts of plasmid DNAs. At 24–48 h after transfection, cells were collected and lysed in 1 ml TNE buffer (10 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP40) containing 0.12 mg ml^{-1} phenylmethyl sulphonyl fluoride, 2 μg ml^{-1} leupeptin, 1 μg ml^{-1} aprotinin, 10 mM NaF and 1 mM Na_3VO_4 on ice. Lysates of equivalent protein content were incubated overnight at 4 °C with anti-pan-Trk polyclonal antibody C14 (Santa Cruz Biotech) (1.5 μg) or anti-VR1 antiserum[12] (1:1,000). Immune complexes were immobilized on protein-A/Sepharose beads, washed with ice-cold TNE, boiled in SDS sample buffer, separated by SDS–PAGE and transferred onto Immobilon-P (Millipore) membranes. Membranes were blocked in TBST buffer (20 mM Tris pH 7.5, 500 mM NaCl, 0.1% Tween-20) containing 5% non-fat milk, then incubated for 2 h at room temperature or overnight at 4 °C in TBST with 1% milk and one of the following primary antibodies: anti-Trk 45 antiserum (1:2,000); anti-VR1 antiserum (1:2,000); or anti-PLC-γ antibody (Upstate Biotech) (0.7 μg ml^{-1}). Membranes were washed with TBST, then incubated with horseradish-peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies. Immunoreactive protein bands were detected by enhanced chemiluminescence using ECL reagents. For western blot analysis of oocyte proteins, 10 cells were lysed in 100 μl of 100 mM NaCl, 1% Triton X-100, 20 mM Tris-HCl pH 7.6 and complete protease inhibitor cocktail (Roche) by drawing 5–10 times each through 15 gauge and 26 gauge needles. Lysates were centrifuged 10 min at 15,000*g* and supernatant fractions were passed twice through 0.22 μm Spin-X filters (Costar). Final flow through (~20 μl) was denatured in SDS sample buffer and analysed as above.

Received 8 February; accepted 18 April 2001.

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

Acknowledgements

We are grateful to W. Neuhausser for providing dissociated DRG neurons, W. Mobley for 2.5 S NGF, D. Shelton for advice regarding NGF administration and J. Poblete and K. Simpson for technical assistance. We thank members of our laboratories for many helpful suggestions and constructive criticism. This work was supported by a NSF predoctoral fellowship (E.P.), NIH predoctoral neuroscience training grant (S.S.), a German Academy of Natural Scientists Leopoldina postdoctoral fellowship (S.-E.J.) and by the NIH (M.V.C., A.I.B. and D.J.).

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Agonist-independent activation of metabotropic glutamate receptors by the intracellular protein Homer

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G-protein-coupled receptors (GPCRs) transduce signals from extracellular transmitters to the inside of the cell by activating G proteins. Mutation and overexpression of these receptors have revealed that they can reach their active state even in the absence of agonist, as a result of a natural shift in the equilibrium between their inactive and active conformations[1]. Such agonist-independent (constitutive) activity has been observed for the glutamate GPCRs (the metabotropic glutamate receptors mGluR1a and mGluR5) when they are overexpressed in heterologous cells[2]. Here we show that in neurons, the constitutive activity of these receptors is controlled by Homer proteins, which bind directly to the receptors' carboxy-terminal intracellular domains[3,4]. Disruption of this interaction by mutagenesis or antisense strategies, or expression of endogenous Homer1a (H1a), induces constitutive activity in mGluR1a or mGluR5. Our results show that these glutamate GPCRs can be directly activated by intracellular proteins as well as by agonists.

When expressed in HEK-293 cells, mGluR1a and mGluR5 display constitutive activity that cannot be inhibited by competitive antagonists[2] or by the non-competitive and neutral mGluR1 antagonist CPCCOEt[5]. This basal activity can be inhibited only by the selective non-competitive antagonists (so-called inverse agonists) BAY 36-7620 (ref. 6) and MPEP[7], respectively. In cultured cerebellar granule cells, which naturally express mGluR1a[2,8], BAY 36-7620 reduces neither basal inositol phosphate formation (Table 1) nor the basal open probability (P_o) of the Ca^{2+}-dependent big K^+ (BK) channel (Fig. 1a, b). These two pathways are activated by mGluR1/5 agonists[9–12], including DHPG[8], the effect of which is antagonized by CPCCOEt (Table 1, Fig. 1a). Although cerebellar granule cells do not express native mGluR5 (refs 2, 8), overexpression of the cloned receptor (mGluR5a) can be obtained after transfection of these cells[8,13]. Even under these conditions, treatment with MPEP decreases neither basal inositol phosphate formation (Table 1) nor basal BK channel activity (Fig. 2a), although the transfected mGluR5a was functionally expressed. It was fully activated by DHPG and this effect was blocked by the competitive neutral mGluR antagonist MCPG (Table 1, Fig. 2a, b). Thus, constitutive activity of native mGluR1a or overexpressed mGluR5a cannot be detected in cultured cerebellar granule cells.

When expressed in HEK-293 cells, the mGluR1a, 5a and 5b splice variants display much higher agonist-independent activity than mGluR1b, 1c and 1d (ref. 2). Interestingly, mGluR1a, 5a and 5b, but not mGluR1b, 1c and 1d, interact with the intracellular Homer proteins[4,14]. We investigated the role of Homer in the activity of mGluR1a and mGluR5a in cultured cerebellar granule cells. These

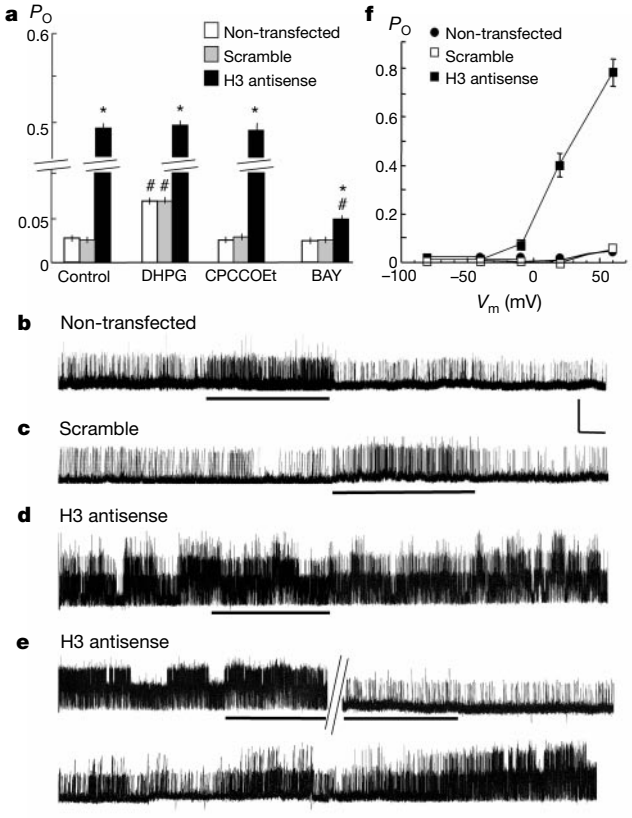

Figure 1 Knock-down of H3 results in constitutive mGluR1a activity. Data obtained from non-transfected neurons or neurons transfected with the indicated oligonucleotides. **a**, BK channel P_o measured in the absence (control) or presence of the indicated drugs. Asterisks and hash symbols, significantly different from non-transfected or control neurons, respectively ($P < 0.01$). **b**–**e**, BK channel recordings. Horizontal bars represent DHPG (**b**–**d**) or BAY 36-7620 (**e**; time lag, 5 min) applications. Calibration bars (5 pA, 10 s) apply to **b**–**e**. **f**, BK channel P_o–voltage relationships (mean ± s.e.m.; $n \approx 10$).