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ORIGINAL ARTICLE

Ca++/CaMKII switches nociceptor-sensitizing stimuli into desensitizing stimuli

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Abstract

Many extracellular factors sensitize nociceptors. Often they act simultaneously and/or sequentially on nociceptive neurons. We investigated if stimulation of the protein kinase C epsilon (PKCε) signaling pathway influences the signaling of a subsequent sensitizing stimulus. Central in activation of PKCs is their transient translocation to cellular membranes. We found in cultured nociceptive neurons that only a first stimulation of the PKCε signaling pathway resulted in PKCε translocation. We identified a novel inhibitory cascade to branch off upstream of PKCε, but downstream of Epac via IP3-induced calcium release. This signaling branch actively inhibited subsequent translocation and even attenuated ongoing translocation. A second 'sensitizing' stimulus was rerouted from the sensitizing to the inhibitory branch of the signaling cascade. Central for the rerouting was cytoplasmic calcium increase and CaMKII activation. Accordingly, in behavioral experiments, activation of calcium stores switched sensitizing substances into desensitizing substances in a CaMKII-dependent manner. This mechanism was also observed by *in vivo* C-fiber electrophysiology corroborating the peripheral location of the switch. Thus, we conclude that the net effect of signaling in nociceptors is defined by the context of the individual cell's signaling history.

Keywords: CaMKII, hyperalgesia, pain, peripheral nociceptive neuron, protein kinase C epsilon, sensitization signaling.

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Progress in understanding molecular mechanisms of pain sensitization is enormous. Many extracellular mediators have been described to act on various classes of receptors including α-s- and α-q-dependent G-protein coupled receptors (GPCR), ligand-gated ion channels, and hormone receptors. This activates a variety of intracellular signaling components, which cause sensitization of peripheral nociceptive neurons (McMahon and Koltzenburg 2005; Hucho and Levine 2007; Basbaum *et al.* 2009; Ren and Dubner 2010). Knowledge of intracellular sensitization signaling is mostly derived from exposure of nociceptive neurons to a single sensitizing stimulus. But in physiological pain, e.g., during inflammation, extracellular mediators are not acting alone. Instead, nociceptive neurons are exposed simultaneously and/or sequentially to a multitude of mediators, which are present for seconds to days and months (Roosterman *et al.* 2006; Ren and Dubner 2010).

Thus, one has to know if sensitizing agents act cooperatively or if they interfere with each other. Besides pain, such context dependence has been shown among others in a macrophage cell line. In this cell line, the combination of two stimuli sometimes is synergistic, but sometimes also

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Abbreviations used: 2APB, 2-Aminoethoxydiphenyl borate; BSA, bovine serum albumin; DMSO, dimethylsulfoxide; DRG, dorsal root ganglion; ESCA, Epac-selective cAMP analogue; GPCR, G-protein coupled receptors; PBS, phosphate-buffered saline; PKC, protein kinase C; TRPV1, transient receptor potential cation channel subfamily V member 1.

antagonistic (Natarajan *et al.* 2006). One cannot simply predict from a single stimulus the result caused by multiple stimuli. Whether or not the result of one-stimulus experiments can be extrapolated to multiple-stimuli situations in peripheral sensory neurons is unknown. Therefore, we set out to test the context dependence of sensitizing stimuli.

We tested for context dependence on the well-documented system of protein kinase C epsilon (PKCε)-dependent sensitization. PKCε is a pivotal intracellular signaling component in various aspects of pain, including mechanical/thermal sensitization, disease- or drug-induced painful neuropathy, and the transition from acute to chronic pain (Khasar *et al.* 1999a; Aley *et al.* 2000; Dina *et al.* 2000; Joseph and Levine 2003; Parada *et al.* 2003; Wang *et al.* 2007, 2011; Eijkelkamp *et al.* 2010; Bogen *et al.* 2012). In nociceptive sensory neurons, PKCε can be activated among others by epinephrine through the prototypic GPCR, β_2-adrenergic receptor. This leads to cAMP production, Epac-mediated phospholipase activation, and PKCε activation (Hucho *et al.* 2005; Wang *et al.* 2011). PKCε-mediated sensitization also can be induced by receptor ligands such as estrogen and G1, both agonists of the novel estrogen receptor GPR30 (Hucho *et al.* 2006; Kuhn *et al.* 2008). One essential step in the activation of PKCε is its translocation to a cellular membrane (Dorn and Mochly-Rosen 2002). In cultures of rat dorsal root ganglion (DRG) neurons, this translocation is transient and returns to baseline within 1.5–5 min (Cesare *et al.* 1999; Hucho *et al.* 2005, 2006; Kuhn *et al.* 2008). Therefore, in principle, if stimulated for a second time, a second translocation event could be observable.

Monitoring the percentage of translocating primary sensory neurons, we asked if PKCε translocates repeatedly if stimulated repeatedly. We identified the molecules regulating context-dependent signaling in nociceptive neurons. Our investigation proceeded from molecular to cellular and living-animal levels. By electrophysiological single-fiber recordings and behavioral experiments, we confirmed the context-dependent changes of sensitization signaling, which we identified in cultured neurons, to be present also in the living animal.

Materials and methods

Ethical statement

Care and use of animals conformed to National Institutes of Health guidelines and the European Communities Council Directive of 24 November 1986 (86/609/EEC), respectively, and were approved by the respective boards.

Chemicals and drugs

(−)-Isoproterenol hydrochloride, Caffeine, dimethylsulfoxide (DMSO), EGTA, Bovine serum albumin, L-Glutamine, Poly-L-Ornithine hydrochloride, DMSO, Paraformaldehyde, and TritonX-100 (Sigma, Taufkirchen, Germany), Ryanodine, ESCA (8-CPT-2′-O-Me-cAMP), IP3 (D-myo-Inositol 1,3,5-Trisphosphate Hexakisacetoxymethyl Ester, 2,4,6-Tri-O-butyryl-), Pluronic, Thapsigargin, UTP, KN-92, KN-93, KN-62, and Autocamtide-2-Related Inhibitory Peptide (AIP) (Calbiochem, Darmstadt, Germany), Collagenase P (Roche, Mannheim, Germany), Trypsin (Worthington Biochemical Corporation, Freehold, NJ, USA), Neurobasal A (without phenol red), B27 supplement, Laminin, minimal essential medium + Glutamax, and Hank's balanced salt solution (Invitrogen, Germany/UK), normal goat serum (Dianova, Hamburg, Germany), Fura-2-AM (cell permeable) (Molecular Probes/Invitrogen), $MgSO_4$ (SERVA, Heidelberg, Germany), EDTA (MERCK, Darmstadt, Germany), and Pen/Strep (Cambrex, East Rutherford, NJ, USA). G1 kindly provided by Dr. E. Prossnitz.

Antibodies

Anti-PKCε rabbit serum kindly provided by Dr. Robert Messing, Alexa-488 chicken anti-rabbit IgG obtained from Invitrogen (Karlsruhe, Germany). FITC anti-rabbit IgG obtained from Dianova.

Animals

Behavioral and cell biological experiments were performed with male Sprague–Dawley rats (200–350 g; Charles River Laboratories, Hollister, CA, USA, and 200–350 g; Harlan, Borchen, Germany, respectively).

DRG cultures

Cultures of dissociated DRG were prepared from male Sprague–Dawley rats as described (Kuhn *et al.* 2008). Cells were plated 0.5 mL/culture (0.5 DRG equivalents per well) in NeurobasalA/B27 media onto polyornithine/laminin-pre-coated glass coverslips and incubated overnight at 37°C in 5% CO_2.

Cell stimulation and immunocytochemistry

Protocol as published (Kuhn *et al.* 2008). Stimulants were mixed in half of the medium of each individual well and added back to the same culture. Negative controls were mock treated. Hydrophobic reagents were dissolved in DMSO (final concentration $\leq$ 0.2%). After stimulation, cells were washed with phosphate-buffered saline (PBS) and fixed with paraformaldehyde (4%, 10 min, room temperature). Fixed cells were permeabilized [0.1% Triton X-100 (10 min, room temperature)], washed three times [0.1% bovine serum albumin (BSA)/PBS, 5 min, room temperature], blocked (5% BSA/10% normal goat serum in PBS, 1 h, room temperature), probed with primary antibodies [1% BSA/PBS, overnight (4°C)], washed (3x 1% BSA/PBS, 5 min, room temperature), and incubated with secondary FITC-coupled antiserum (final concentration 1 : 500, 1 h, room temperature). After three final washes (PBS, 5 min, room temperature), the cultures were mounted with Fluoromount-G (Southern Biotech/Biozol, Birmingham, AL, USA) containing DAPI (5 μg/mL).

Evaluation of PKCε translocation

Cells were evaluated using a 63x oil-immersion objective on a Zeiss Axioplan 2 microscope (Zeiss, Oberkochen, Germany). Per culture, fifty randomly selected neurons were evaluated for PKCε-translocation, i.e., enrichment of PKCε at the plasma membrane resulting in a clear rim-like structure. In contrast, cells were considered as non-translocating if the immunofluorescent signal faded toward the

plasma membrane. The evaluating scientists were blinded in respect to the treatment condition of the respective cultures. Data are plotted as mean percentage of translocating cells. No more than two cultures treated alike are from the same animal. All experiments have been repeated on at least two separate days. Images were taken using a confocal microscope Zeiss LSM 510 [63x oil objective, evaluation with Image J (NIH, Bethesda, MD, USA)].

Calcium imaging

Overnight cultures of DRG neurons were incubated with Fura-2-AM (1 μM, 30 min, 37°C, 5% CO_2) in Neurobasal A media. After washing 3x 10 min with Neurobasal A media (37°C, 5% CO_2), responses to isoproterenol (1 μM, 60 s, flow through) were recorded using a Leica DM IRE2 microscope [(Solms, Germany) imaging software Simple PCI 6 (Hamamatsu Photonics, Iwata, Japan)]. Paired images of 340/380 nm excitation wavelength were taken every 2 s. Viability of the cells was assured by final 30 mM KCl exposure. Only cells with stable baseline and KCl-induced responses were evaluated.

Combined calcium imaging and PKCε translocation in the same cell were performed on a BD Pathway 855 High-Content Bioimager. Overnight cultures of rat DRG neurons in 96-well plates were loaded with Fura-2-AM as above. Cellular calcium was monitored as above using a 20x objective in a controlled environment at 37°C, 5% CO_2. After a test pulse of 5 μL Neurobasal A media (1 μL/s), calcium fluxes were monitored for 30 s. Then, isoproterenol was added (5 μL, f.c. 1 μM, 1 μL/s) and fluxes monitored for 40 s. Cells were fixed [10 min, 8% PFA added directly to the well (4% final concentration)]. Immunocytochemistry was performed as described above. Cells, which showed calcium influx, were revisited and evaluated for PKCε translocation using a 20x objective [(images analyzed with Image J (NIH)].

Single fiber *in vivo* electrophysiology

Single-fiber recordings were performed as described (Chen and Levine 1999). Recordings were made from the saphenous nerve of anesthetized animals (sodium pentobarbital (50 mg/kg intraperitoneal), additional doses given throughout the experiment to maintain areflexia). Bipolar stimulating electrodes were placed under the nerve distal to the recording site. The nerve was cut proximal to the recording site. Fine fascicles of axons were dissected from the nerve and placed on a recording electrode. Single units were detected by electrical stimulation of the nerve. Conduction velocities of fibers below 2 m/s were classified as C-fibers. Receptive fields of individual C-fibers were located by mechanical search stimuli (a blunt probe with smooth tip). The fiber was determined as cutaneous if lifting and stimulating the skin activated it and/or if the receptive fields moved when the skin was moved relative to subcutaneous tissue. These cutaneous C fibers were verified by the latency delay technique (electrically evoked spikes result in longer latency than if mechanically stimulated). Mechanical threshold was determined with calibrated Von-Frey-hairs and defined as the lowest force that elicited at least two spikes within 1 s, in at least 50% of trials. Neural activity was recorded using an IBM compatible computer with micro 1401 interface (CED, Cambridge, UK), and further analyzed off-line using Spike2 software (CED). Isoproterenol and/or Epac were injected in concentrations and volumes as in the behavioral experiment adjacent to the receptive field of an identified C fiber. Mechanical threshold was measured 15 min after the injection.

Testing of mechanical nociceptive threshold in the rat

As published (Dina *et al.* 2003; Hucho *et al.* 2006), the nociceptive flexion reflex was quantified with a Randall–Selitto paw pressure device (Stoelting, Wood Dale, IL, USA), which applies a linearly increasing mechanical force to the dorsum of a rat's hind paw. The nociceptive mechanical threshold was defined as the force in grams at which the rat withdrew its paw.

Baseline paw-withdrawal threshold was defined as the mean of six readings before test-agent injection. Each paw was treated as independent, and each experiment was performed on a separate group of rats. Each group of rats was treated with the agonists and/or inhibitors injected intradermally on the dorsum of the hind paw. Measurement of nociceptive threshold was taken 30 min after administration. Inhibitors were injected as described previously (Khasar *et al.* 1999a).

Statistical analysis

All statistical comparisons were made with one-way ANOVAs followed by Dunnett's test for comparisons with one control, or Tukey–Kramer post hoc test for multiple comparisons; exception Fig. 5f–h, where repeated-measure ANOVA was performed. $p < 0.05$ is considered as statistically significant. Asterisks indicate range of p values ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$). Throughout this manuscript, error bars indicate standard error of the mean, SEM.

Results

First stimulus blocks effect of second stimulus

PKCε-dependent nociceptor sensitization has been described using β-adrenergic receptor agonists as a model system (Khasar *et al.* 1999a, 2008; Hucho *et al.* 2005; Wang *et al.* 2011). We stimulated sensory neurons with the β-adrenergic receptor-specific agonist isoproterenol (for overview of pharmacological treatments see Fig. 6). As reported previously (Hucho *et al.* 2005), stimulation with isoproterenol for 30 s resulted in translocation of PKCε to the plasma membrane in a subgroup of about 15–20% of DRG neurons (Fig. 1a and b). Thus, in isoproterenol-treated cultures, there were non-translocating neurons (Fig. 1a, left) and translocating neurons (Fig. 1a, middle). The percentage of neurons showing a translocation is the read out which is monitored in the following cellular experiments.

We tested if PKCε translocates in nociceptive neurons, a subgroup of sensory neurons involved in the detection of painful stimuli. Previously, we described PKCε to be activated in nociceptive neurons labeled by the isolectin IB4 (Hucho *et al.* 2005; Kuhn *et al.* 2008). Others found PKCε to regulate the sensitivity of nociceptive neurons defined by the expression of the transient receptor potential cation channel, subfamily V, member 1 (TRPV1) (Cesare *et al.* 1999; Bhave *et al.* 2003; Amadesi *et al.* 2006; Mandadi *et al.* 2006). We therefore now tested if isoproterenol induces PKCε-translocation in TRPV1-expressing nociceptive neurons. Double labeling of isoproterenol-stimulated neurons showed TRPV1 to be expressed in 87.5 ± 4.7% of PKC-translocating neurons (Fig. 1c).

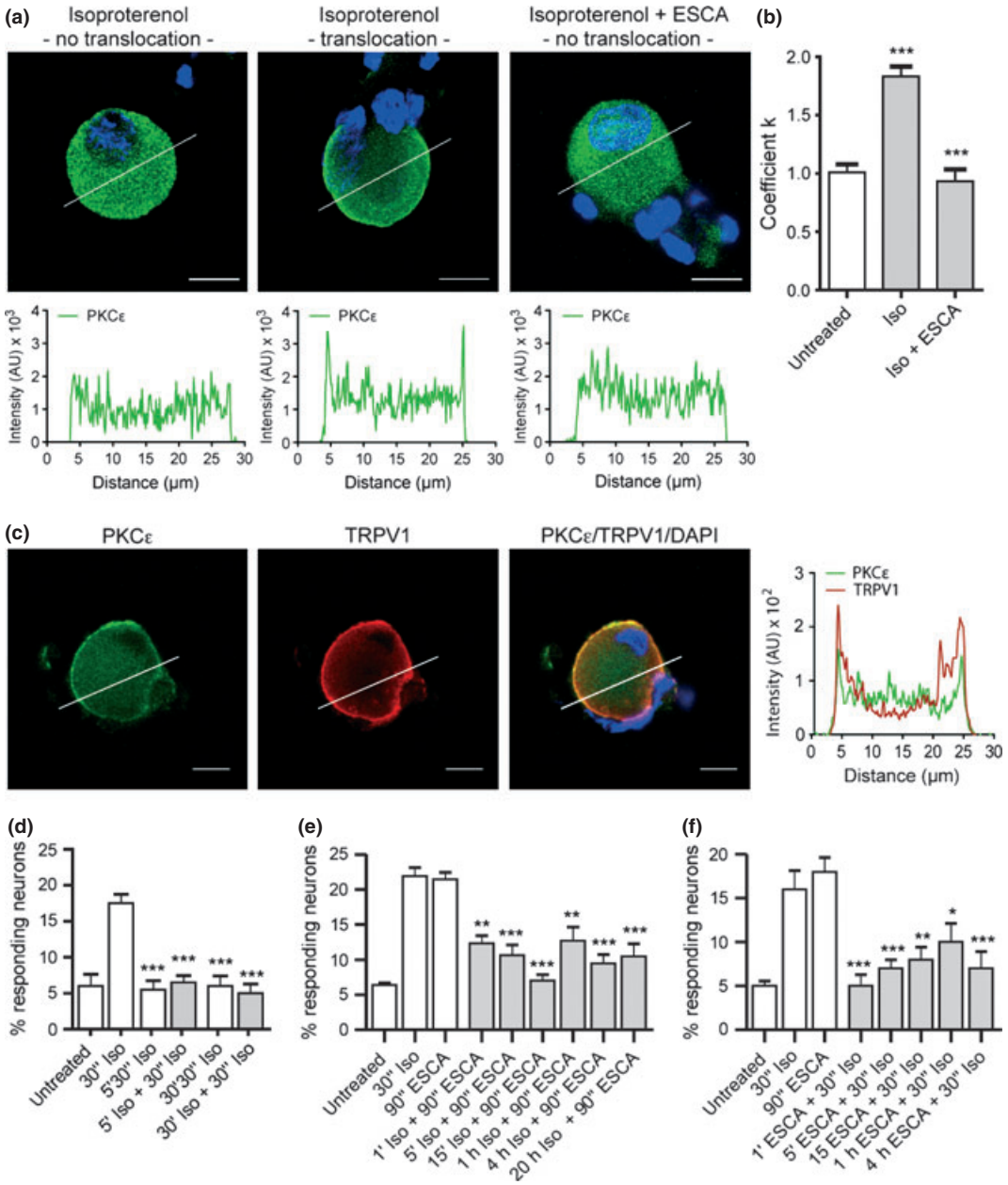

Fig. 1 Sensitizing signaling initiates long-lasting inhibition of protein kinase C (PKC)ε translocation. (a) The β-AR agonist isoproterenol (Iso, 1 μM, 30 s) and the Epac-activator ESCA (10 μM, 90 s) translocated PKCε in a subgroup of cultured sensory neurons toward the plasma membrane. Stimulation with Iso (30 s) and then with the Epac-selective cAMP analogue (ESCA) (90 s) did not result in observable translocation (scale bar equals 10 μm). (b) Quantification of PKCε translocation as shown in (a) by dividing the fluorescence intensity of the plasma–sub plasma membrane region and the center of the cell as measured along a confocal line scan [Iso (1 μM), $k = 1.83 \pm 0.19$; Iso + ESCA (10 μM), $k = 0.93 \pm 0.23$, $p < 0.001$]. (c) Stimulation with Iso (1 μM) showed translocation in TRPV1-expressing cells. A total of 87.5 ± 4.7% of PKCε-translocating neurons expressed also TRPV1 ($n = 300$). (d) Stimulation with Iso (1 μM) showed translocation at 30 s (the previously reported peak of translocation) and no translocation at later time points (5.5 min, 30.5 min), independent of whether stimulated once or a second time cells fixed 30 s after last stimulus application, for positive and negative controls see white bars, number of evaluated cultures: $n = 6$, $p < 0.001$ (compared to white bar Isocontrol)]. (e) Pretreatment with Iso (1 μM) with intervals ranging from 1 min to 20 h abolished ESCA (10 $μM)-induced translocation of PKCε [$n = 6$, asterisks indicate statistical significance (*$p < 0.05$, **$p < 0.01$. ***$p < 0.001$, comparison to white bar positive controls)]. (f) Also inverse order of stimulation (first ESCA, then Iso) abolished PKCε translocation ($n = 4$, asterisks indicate statistical significance (comparison to white bar positive controls), no statistical difference to untreated controls). Variance in all figures is given as SEM.

We reported previously that the translocation of PKCε to the plasma membrane is transient and returns to homogenous cytoplasmic distribution within 1.5–5 min (Hucho *et al.* 2005). This fast return to homogenous cytoplasmic distribution allows, therefore, to test if also a second isoproterenol stimulus results in a second translocation event.

In accordance with our previous publication (Hucho *et al.* 2005), we observed translocation of PKCε to the plasma membrane within seconds after isoproterenol application, but not at time points beyond 5 min (Fig. 1d).

Finally, to test for the context dependence of PKCε translocation, we added a first isoproterenol stimulus and applied isoproterenol for a second time after 5 and 30 min, respectively. Fixing the cells after additional 30 s (that is 5.5 min and 30.5 min after the application of the first stimulus, respectively), the second stimulus did not induce a second translocation (Fig. 1a, b and d).

Inhibition of translocation is not caused by receptor desensitization

Several receptor-desensitization mechanisms are known. They occur usually within a few seconds, last for minutes, and result in attenuation of ligand-induced intracellular signaling. Such a mechanism might underlie the lack of effect of a second isoproterenol application (Lohse *et al.* 1990; Daaka *et al.* 1997). To exclude receptor desensitization, we therefore next attempted to induce translocation first by activating the receptor with isoproterenol and then to activate the downstream signaling component, Epac, with the membrane-permeable 'Epac-selective cAMP analogue' [ESCA (also referred to as 'CPTOMe' or '007′)] (Rehmann *et al.* 2003; Hucho *et al.* 2005).

As described earlier, treatment with ESCA alone resulted in PKCε translocation similar to isoproterenol stimulation (peak of translocation was reported to be at 90 s, (Hucho *et al.* 2005), Fig. 1e). Adding ESCA for additional 90 s after an initial isoproterenol treatment, we could not observe a second PKCε-translocation event (Fig. 1a, b and e). This was true for interstimulus intervals of 1 min to 20 h. We could never detect a second translocation. Thus, our results showed that the block of a second PKCε translocation is located downstream of the receptor. It also showed that the block of a second PKCε translocation is fast (within 1 min) and, in contrast to common receptor-desensitization processes, rather long lived [over 20 h, Fig. 1e (longest observable time point with this model system)].

The inhibition of a second translocation is initiated at Epac or downstream of it

Next, we attempted to narrow down where the blocking signaling is initiated. We tested if it is initiated upstream or downstream of ESCA-activated Epac. Therefore, we changed the order of stimulation. We first stimulated Epac with ESCA and then the receptor with isoproterenol. If the block of a PKCε translocation is initiated upstream of Epac, then a second induction of translocation should now be possible.

This order of stimulation, too, resulted in blocking of a second PKCε translocation (Fig. 1f). Thus, the long-term inhibitory signaling cascade counteracting PKCε translocation is activated at or downstream of Epac.

A second stimulation abolished an ongoing sensitization signaling

When switching the order of stimulation from Isoproterenol/ESCA to ESCA/Isoproterenol, the shortest time interval revealed an additional aspect. There, Isoproterenol was added 60 s after the addition of ESCA and stimulation continued in the presence of both substances for further 30 s. Thus, the cultures were fixed after a total of 90 s of ESCA stimulation, including a total of 30 s of isoproterenol stimulation. At this time point, both ESCA and isoproterenol should have induced maximal translocation. But, we could not detect any significant PKCε translocation (Fig. 1f).

This suggests that the ESCA-initiated PKCε translocation is abrogated by the application of the second stimulus, isoproterenol. Thus, potentially a second stimulus is not simply blocked. Instead, the second stimulus resulted in active signaling. This signaling was potentially then rerouted from a translocation-initiating to a translocation-abrogating signaling. In the following experiments, we investigated this hypothesis further in detail.

Inhibitory signaling is separable from sensitizing signaling

To further characterize this inhibitory signaling, we mapped the point in the cascade where the inhibitory signaling is initiated. We had found the inhibitory signal to be derived from signaling at or downstream of Epac (see above). Branching might occur at the level of phospholipase C, which produces diacylglycerol and IP3, the former activating PKC whereas the latter induces calcium release from internal calcium stores (Hucho *et al.* 2005). PKCε is a 'novel PKC' lacking a calcium-binding domain. Thus, we considered an IP3-induced calcium release unlikely to modulate PKCε activation (Parekh *et al.* 2000). Nevertheless, to exclude this signaling branch, we inhibited the IP3 receptor with 2-Aminoethoxydiphenyl borate (2APB). Surprisingly, we observed a reversal of the PKCε-translocation block. Thus, in presence of 2APB, PKCε translocation was observed also after a second stimulation (Fig. 2a).

2APB has been reported to have various effects in addition to blocking IP3 receptors (Parekh and Putney 2005). Thus, the mechanism underlying the effect of 2APB had to be clarified. If indeed the block of the IP3 receptor enabled a second PKCε translocation, then in turn the activation of the IP3 receptor should block PKCε translocation. We used a membrane-permeable IP3 analogue to activate the IP3 receptor. Indeed, pre-treatment with IP3 led to blockade of PKCε translocation in response to ESCA and ESCA/Iso stimulation (Fig. 2b). This corroborates the result of the 2APB-pre-treatment

experiment and suggests IP3 and the IP3 receptor to be involved in the rerouting of sensitizing signaling.

Activation of P2Y receptors block subsequent PKCε translocation

To further confirm that the IP3/calcium signaling branch induces the block of subsequent PKCε translocation, we tested if also IP3 produced endogenously after receptor activation would lead to abolishment of Epac-initiated PKCε translocation. Metabotropic α-q-coupled P2Y receptors are activated by UTP and result in IP3 production in primary nociceptive neurons (Ralevic and Burnstock 1998; Sanada *et al.* 2002; Gerevich and Illes 2004). And indeed, as predicted by our previous results, 30-min pre-treatment with UTP abolished the induction of PKCε translocation completely (Fig. 2c).

IP3 and ryanodine receptor mediate the inhibition of PKCε-translocation

IP3 receptors regulate calcium influx from intracellular stores and from extracellular space, and are involved in calcium-independent signaling (Bolotina 2004; Dellis *et al.* 2006). Not only IP3 receptors, but also ryanodine receptors mediate rise of cytoplasmic calcium. If our reasoning is correct and a rise of intracellular calcium is essential for the inhibition of PKCε translocation, then also raising intracellular calcium via the ryanodine receptor should abolish PKCε translocation. To test this, we pre-treated primary DRG cultures for 30 min with ryanodine, a ryanodine-receptor activator, or with caffeine, a ryanodine-receptor activator, and concomitant IP3-receptor inhibitor. Both treatments blocked ESCA-induced PKCε translocation (Fig. 2d). Thus, indeed, the PKCε block appears to depend not exclusively on IP3 receptor-mediated calcium rise, but can also be mediated by other calcium channels such as the ryanodine receptor.

Rise of intracellular calcium causes inhibition of PKCε translocation

IP3-mediated activation of the IP3 receptor results in a rapid increase of intracellular calcium. Therefore, to further corroborate our pharmacological results, we next tested whether isoproterenol treatment leads to a rise in intracellular calcium. Furthermore, we tested if this calcium change occurs in cells translocating PKCε, and if this calcium change is essential for the inhibition of translocation.

Using Fura-2-based calcium imaging, we found that neurons react to isoproterenol with a pronounced change in the concentration of cytoplasmic calcium (Fig. 3a and c). PKCε translocation by sensitizing stimuli such as epinephrine, isoproterenol, estrogen, and GPR30-specific derivatives of estrogen occur only in about 15% of all sensory neurons in culture (difference between unstimulated and stimulated cultures) (Hucho *et al.* 2005; Kuhn *et al.* 2008). Correspondingly, stimulation with isoproterenol resulted in calcium influx in 16.7% of the cultured neurons.

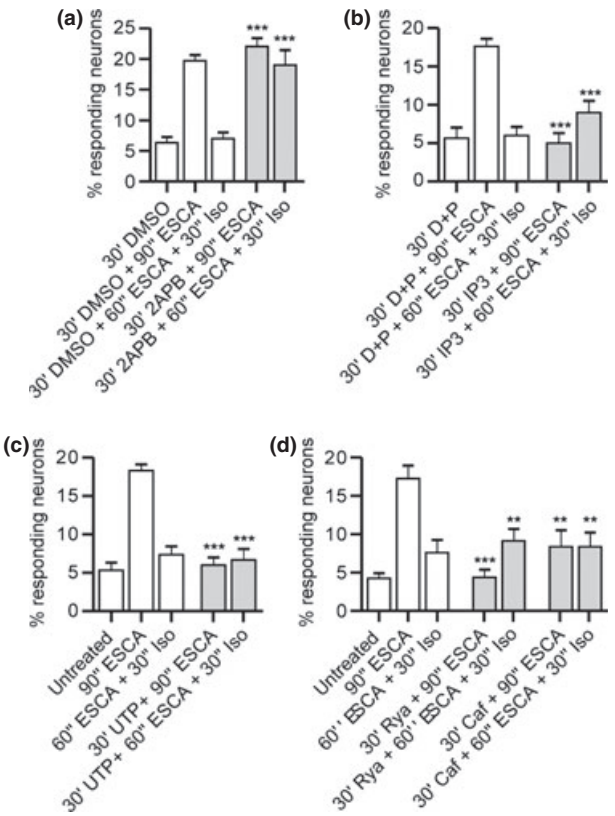

Fig. 2 Protein kinase C (PKC)ε-translocation-inhibiting pathway branches off via IP3 and can be activated pharmacologically as well as through endogenous receptor-mediated processes. (a) Dimethylsulfoxide (DMSO) in the concentration used (f.c. in culture 0.2%) did not affect PKCε translocation after Epac-selective cAMP analogue (ESCA) (10 μM) and Iso (1 μM) stimulation. Also pre-treatment with the DMSO-solved IP3R blocker, 2-Aminoethoxydiphenyl borate (2APB) (178 μM), alone did not interfere with Iso and ESCA single-stimulus induction of PKCε translocation. But, PKCε translocation was observable in cultures stimulated with both stimuli (ESCA and Iso) if pre-treated with 2APB (number of evaluated cultures: $n = 6$, asterisks indicate statistical significance [comparison to white bar 30' DMSO control)]. (b) Activation of the IP3 receptor with IP3 (100 μM) in its solvent DMSO + Pluronic (D+P) (f.c. in culture 0.02%) abolished translocation in response to a first (ESCA, 10 μM) as well as subsequent second stimulus [Iso, 1 μM, number of evaluated cultures: $n = 6$, asterisks indicate statistical significance (comparison to white bar positive controls)]. (c) UTP (100 μM)-mediated activation of the P2Y receptor, which produces endogenously IP3, resulted in block of translocation [number of evaluated cultures: $n = 6$, asterisks indicate statistical significance (comparison to white bar positive controls)]. (d) Translocation was abolished by pre-treatment with the ryanodine receptor agonists, Ryanodine (10 μM) or caffeine (10 mM), the latter being also an IP3R inhibitor [number of evaluated cultures: $n = 8$, asterisks indicate statistical significance (comparison to white bar positive controls)].

We next tested if the change of intracellular calcium can be observed not only in a similar percentage of neurons but in the very same cells as the PKCε translocation. We performed

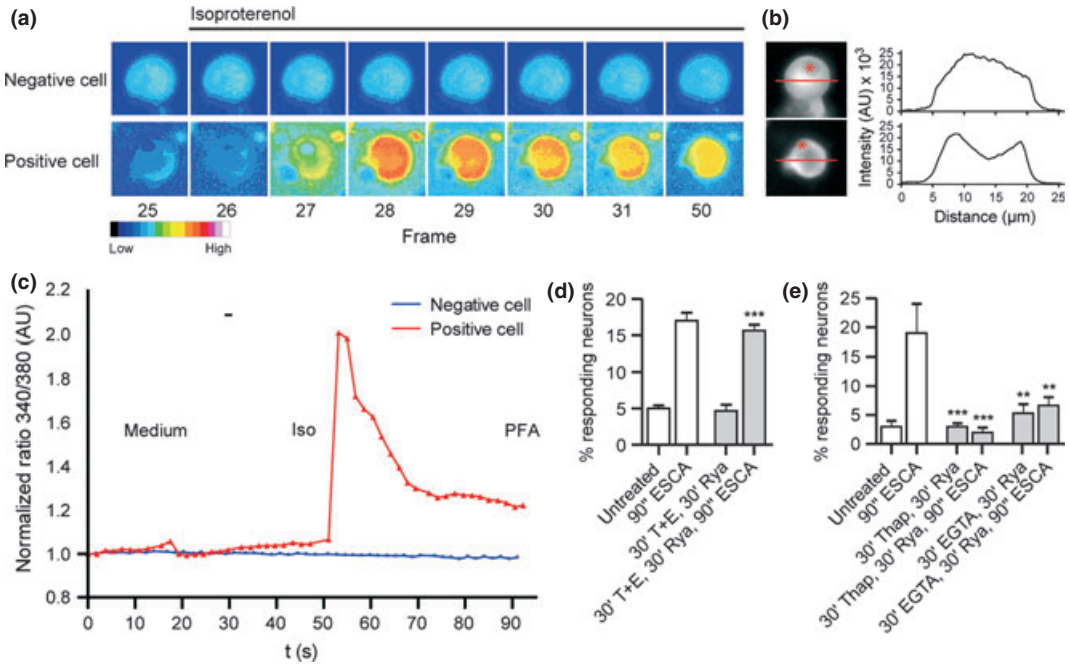

Fig. 3 Rerouting switch is calcium dependent. (a) Isoproterenol stimulation resulted in an increase of intracellular calcium in 16.7% of the neurons corresponding well with the detected 15% of protein kinase C (PKC)ε-translocating neurons. Shown are representative images of Fura-2 loaded Iso-responding 'positive' and Iso-non-responding 'negative' cells. The 340/380 nm ratio has been converted into false colors. Images were taken approximately every 2 s (numbers underneath indicate image number). (b) Calcium rise was found to occur in PKCε-translocating neurons (epifluorescent images of PKCε immunfluorescence of the same two cells as in (a), above. Intensity histograms along the indicated red lines are presented. The red star indicates the nuclei of the cells). (c) Exemplary traces of responsive (red symbols) and non-responsive (blue symbols) rat dorsal root ganglion (DRG) neurons shown in (a) and (b). Arrows indicate the test pulse and the addition of Iso as well as of PFA. (d) Pre-treatment with Thapsigargin plus EGTA (T + E, 1 μM and 2.5 mM, respectively) to deplete intracellular calcium stores as well as extracellular calcium abolishes ryanodine-induced block of PKCε translocation [number of evaluated cultures: $n = 6$, asterisks indicate statistical significance (comparison to white bar untreated controls)]. (e) Neither treatment with Thapsigargin nor with EGTA alone abolished Ryanodine-induced block of PKCε translocation [number of evaluated cultures: $n \geq 4$, asterisks indicate statistical significance (comparison to white bar positive controls)].

live-cell calcium imaging followed by fixation 30 s after isoproterenol application and evaluated the very same cells for PKCε translocation. Indeed, in DRG neurons with isoproterenol-induced calcium rise, we also observed PKCε translocation (Fig. 3a and b).

To test directly if calcium underlies the inhibition of PKCε translocation, we depleted calcium from both internal as well as external sources by thapsigargin and EGTA exposure. Indeed, in the absence of a calcium rise, ryanodine no longer induced a block of PKCε translocation (Fig. 3d). Neither EGTA nor thapsigargin alone abolished the ryanodine effect, indicating that calcium from either intracellular or extracellular sources is sufficient for the block of PKCε signaling (Fig. 3e).

Finally, we investigated if an increase of intracellular calcium not only abolishes future but also ongoing translocation. We added the inducers of calcium release from intracellular calcium stores, ryanodine, IP3, as well as UTP, overlapping for 60 s with ESCA. Indeed, as with the overlapping isoproterenol signaling presented above (Fig. 1f), the already started ESCA-induced translocation was completely abolished (Fig. 4a).

CaMKII inhibitors abolish the switch to translocation-inhibiting signaling

We showed that the rise of intracellular calcium is a prerequisite for the change from PKCε translocation to PKCε-translocation-inhibitory signaling. Now, we attempted to characterize an effector of calcium, which initiates the block. One calcium-dependent component central to long-lasting changes in neurons is CaMKII (Silva *et al.* 1992).

If CaMKII indeed is activated by the rise in calcium, and if this change indeed is central for the long-lasting inhibition of a second translocation, then blockers of CaMKII should attenuate this effect. Consequently, in the presence of blockers of CaMKII, translocation of PKCε should be observable also after application of the second stimulus.

We tested the CaMKII inhibitors, KN93, KN62, and the CaMKII-inhibitory peptide, AIP. Indeed, the percentage of PKCε-translocating neurons in neurons pre-treated with any

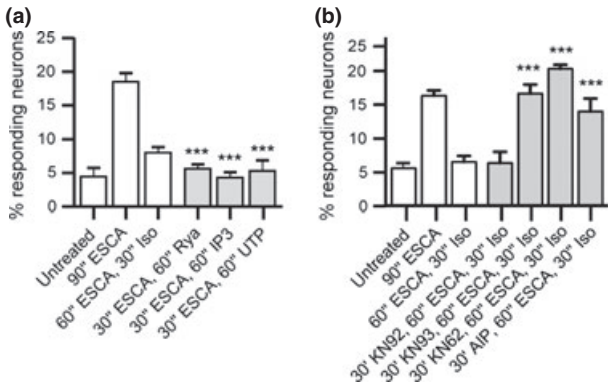

Fig. 4 The rerouting reverses ongoing sensitizing signaling. (a) Stimulation of calcium release by ryanodine (10 μM), IP3 (100 μM), or UTP (100 μM) reversed ongoing sensitization signaling leading to protein kinase C (PKC)ε translocation even if applied after Epac-selective cAMP analogue (ESCA) treatment [number of evaluated cultures: $n > 4$, asterisks indicate statistical significance (comparison to white bar positive controls)]. (b) The CaMKII inhibitors KN-93 (10 μM), KN-62 (5 μM), AIP (1 μM), but not the control compound KN-92 (10 μM) abolished the inhibitory effect of ryanodine on PKCε translocation [number of evaluated cultures: $n > 6$, asterisk indicates statistical significance (comparison to white bar untreated, ESCA, Iso controls)].

of these three inhibitors was as high as in the single-stimulus controls (Fig. 4b). For the inhibitor KN93, an inactive control compound, KN92, is available. As expected, the control compound KN92 did not affect the inhibition of the second translocation. In conclusion, CaMKII mediates the rerouting of sensitizing signaling to translocation inhibition.

Switch to inhibitory signaling erases mechanical hyperalgesia in animals

Next, we verified that the inhibitory signaling of a second stimulus is also present in the living animal, thereby reversing an already established hyperalgesia. We injected isoproterenol intradermally into the hind paw of adult male rats at a concentration known to induce PKCε-dependent mechanical hyperalgesia [measured by the Randall–Selitto paw pressure test (Khasar *et al.* 1999a, b)]. After 30 min, when robust hyperalgesia was detectable, the second stimulant (ESCA) was injected. As in the cellular experiments, this second stimulus completely reversed the sensitization (Fig. 5a).

We confirmed this effect with another sensitizing stimulus, with the estrogen derivative specific for GPR30, G1 (Hucho *et al.* 2006; Kuhn *et al.* 2008). G1-induced mechanical hyperalgesia was completely reversed by a subsequent ESCA injection (Fig. 5b). Thus, also in the animal, the switch to sensitization-inhibiting signaling was not restricted to the β_2-adrenergic receptor.

If the reversal of hyperalgesia is based on the same mechanism as shown in the cellular experiments, one would predict the results of the following three experiments: Ryanodine should (i) block the development of hyperalgesia, and (ii) reverse already established hyperalgesia; and iii) CaMKII inhibitors should abolish the inhibitory effect of isoproterenol given as second stimulus.

Ad (i) Ryanodine injected alone had no effect on the mechanical nociceptive threshold in the rat. But, when injected 30 min before ESCA, ryanodine completely attenuated ESCA-induced mechanical hyperalgesia (Fig. 5).

Ad (ii) If injected after ESCA, ryanodine completely reversed mechanical hyperalgesia (Fig. 5d).

Finally, ad (iii): We pre-treated the rat paw by intradermally injecting the CaMKII inhibitor KN93 at concentrations as low as 10 ng/2.5 μL. This pre-treatment completely blocked the isoproterenol-induced reversal from ESCA-induced hyperalgesia to baseline sensitivity (Fig. 5e).

To go beyond the hyperalgesic model used in this investigation, we tested if the switch to inhibitory signaling abolishes hyperalgesia also in a more complex model of mechanical hyperalgesia. Indeed, activation of the rerouting switch by ryanodine completely attenuated the mechanical hyperalgesia induced by Carrageenan-evoked inflammation (Fig. 5f).

In conclusion, antihyperalgesia in the living animal appears to rely on the switch/rerouting of intracellular signaling, which we described on a cellular level. Thus, prevention of sensitization can be accomplished through activation of an endogenous signaling cascade distinct from the sensitizing PKCε pathway.

Prevention of sensitization in single-fiber recordings – a peripheral mechanism

Our results indicate that sensitivity can be steered by stimulation of a rerouting switch in sensory neurons. To confirm that this is a peripheral mechanism, we recorded the reversal of sensitization electrophysiologically directly from single afferent nerve fibers in living animals. The nerves are cut between the site of recording and the spinal cord. Thus, any change of action-potential response arriving from the periphery can only be modulated by peripheral tissue, not by the spinal cord. Injected into the receptive field on the hind paw of the respective nerve fiber, isoproterenol as well as ESCA, each resulted in increased sensitivity to mechanical stimuli as tested with Von-Frey-hairs (Fig. 5g and h). However, apparently a first sensitization switched the signaling so that a subsequent second stimulus was rerouted and reversed the already established sensitization (Fig. 5h).

Discussion

Here, we describe a novel endogenous intracellular inhibitory mechanism regulating hyperalgesia. We show that the signaling pathway toward PKCε and thus toward PKCε-dependent hyperalgesia branches and activates a so far not described inhibitory pathway. This inhibitory pathway is located downstream of known negative feedback loops of

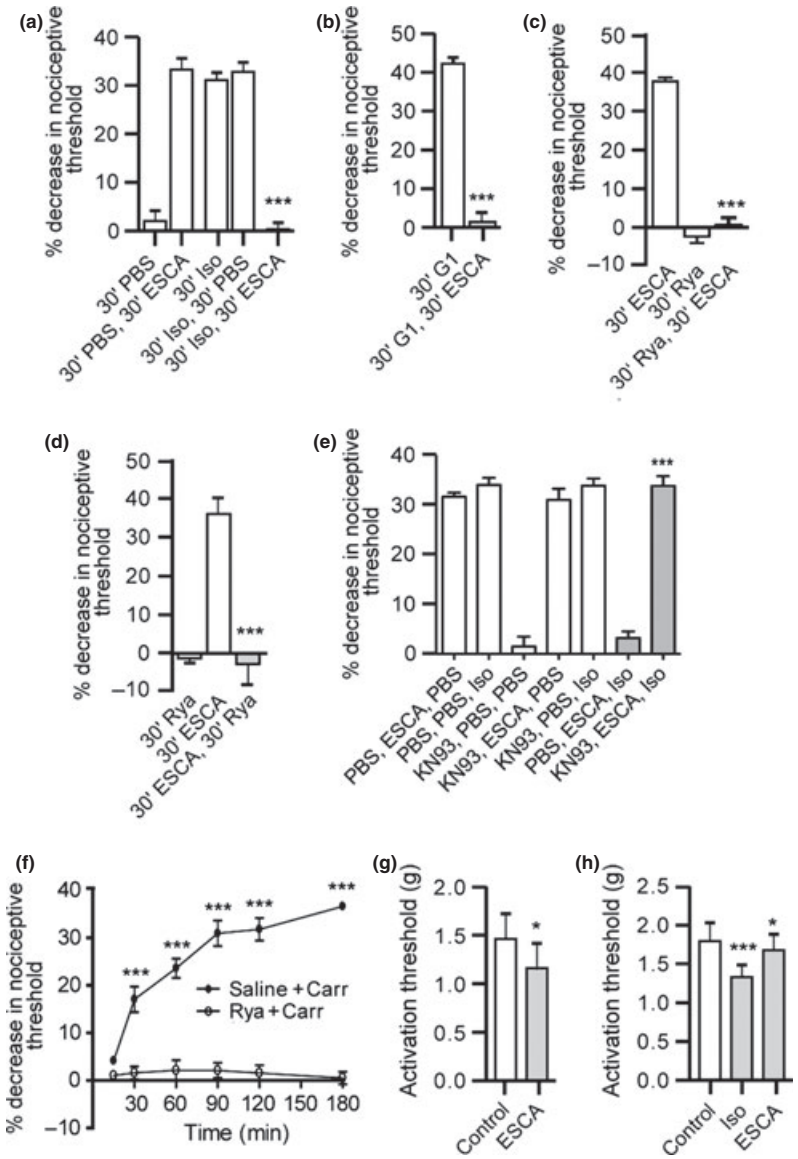

Fig. 5 Reversal of hyperalgesia through second sensitiziation stimulus in rat behavior - a peripheral mechanism. (a) Injection of isoproterenol (1 μg/2.5 μL) or Epac-selective cAMP analogue (ESCA) (6.3 μg/2.5 μL) intradermally into hind paws of male rats resulted in robust mechanical hyperalgesia after 30 min. The hyperalgesia fully established 30 min after the initial isoproterenol injection was reversed back to baseline if followed by ESCA injection [measurement taken 30 min after the ESCA injection, $n > 8$, asterisks indicate statistical significance (comparison with white positive controls)]. (b) Successive stimulation reversed mechanical hyperalgesia also if induced through the estrogen derivative G1 acting on a different G-protein coupled receptor (GPCR), GPR30 than isoproterenol [$n = 6$, asterisks indicate statistical significance (comparison with white positive control)]. (c) Pre-treatment with ryanodine (2.5 μg/2.5 μL) attenuated ESCA-induced hyperalgesia [asterisks indicate statistical significance (comparison with white bar positive controls)]. (d) Activation of the inhibitory module with ryanodine reversed ESCA-induced hyperalgesia [$n > 8$, asterisks indicate statistical significance (comparison with white bar positive controls, $p > 0.05$ comparison ryanodine vs. ESCA/ryanodine treatment)]. (e) Injection of the CaMKII inhibitor, KN-93 (10 ng/2.5 μL) blocked completely the switch of the second stimulus (Iso) into an antinociceptive reagent. Thus, the hyperalgesia induced by ESCA injection was maintained even after the injection of isoproterenol ($n > 4$). (f) Injection of ryanodine (2.5 μg/2.5 μL) blocked inflammatory mechanical hyperalgesia induced by injection of carrageenan (1%, 2.5 μL, 15 min after ryanodine, ryanodine + carrageenan $n = 6$, saline + carrageenan $n = 4$, asterisks indicate statistical significance). (g) Intradermal injection of ESCA resulted in mechanical hyperalgesia as measured by single-fiber recordings in the living animal in response to Von-Frey-hair mechanical stimulation (7 of 12 fibers reduced their threshold, mean of all 12 are shown, $p = 0.014$). (h) Injection of ESCA into isoproterenol-sensitized skin reversed the hyperalgesia [12 of 23 fibers sensitized by isoproterenol (52%), 7 of 12 sensitized fibers returned to baseline (58%), mean of all 23 fibers shown for all three conditions, asterisks indicate statistical significance (comparison with white bar positive control)].

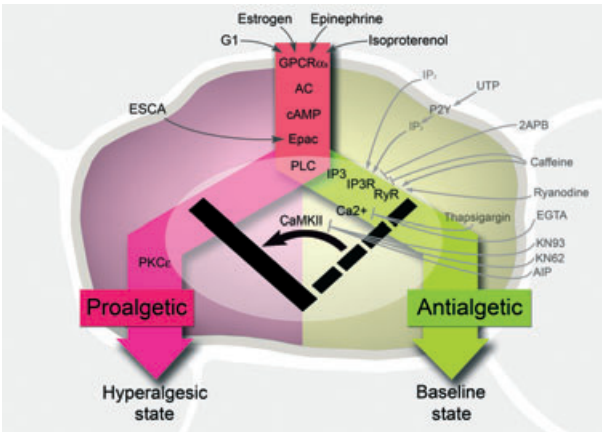

Fig. 6 Calcium and CaMKII rule over the switch from pro- to antialgetic signaling. Activation of α-s-coupled GPCRs initiates proalgetic signalling (red arrow) via adenylyl cyclase (AC), cAMP, Epac, phospholipase C (PLC) and protein kinase C (PKCε), resulting in hyperalgesia. We now found in addition PLC to activate via IP3 a switch (black bold lines underlined by white sphere). Thereby, the signaling initiated by subsequent stimulation of the Epac pathway is rerouted to the antialgetic signaling (green arrow) reversing already established hyperalgesia. The rerouting switch can be also activated through pharmacological stimulation of the IP3 receptor (IP3R) or the ryanodine receptor (RyR) as well as through α-q-coupled GPCRs such as the P2Y ATP/UTP receptor. Substances blocking calcium release or calcium calmodulin-dependent kinase CaMKII also block the switch to the antialgetic signaling (see substances in light grey). The positioning of the switch represents two complementary computation states of one and the same cell: either 'sensitizing' stimuli induce sensitization of nociceptive neurons or they are 'interpreted' by the neuron as antialgetic thereby erasing already established hyperalgesia. Thus, the result of stimulation is not only dependent on the proteins expressed but also on the signaling context of each single cell.

GPCRs, such as the G-protein switch, β arrestin coupling/signaling, or internalization (Lohse *et al.* 1990; Daaka *et al.* 1997). In contrast to known GPCR regulation mechanisms, which over a few minutes result in fading of newly initiated signaling such as cAMP production, the signaling characterized here seems not to be a simple negative feedback loop. Instead, prior stimulation determines not only the outcome of future sensitizing stimuli quickly (1 min), but also long-term (investigated up to 20 h) outcomes.

We found the same mechanism not only to inhibit future stimulation of sensitizing PKCε signaling but to reverse also ongoing as well as past sensitization. Interestingly, this antihyperalgesic action can be separated from the sensitizing signaling cascade. Thus, pharmacologically sensitization signaling could be reverted in cell culture and hyperalgesia inverted in the living animal. Interestingly, desensitization was accomplished not by the blocking of signaling, but by the initiation of endogenous signaling cascades. Our data suggest that, thereby, the signaling was switched from a sensitization to a desensitization pathway. For the switch to desensitization signaling, an increase of intracellular calcium and the activation of CaMKII is essential. As an endogenous mechanism in the periphery is targeted, and as the observed inhibition of PKCε translocation lasts for at least 20 h, the sensitization-rerouting switch could be a powerful and conceptually novel way for therapeutic intervention.

Several aspects of calcium and CaMKII in hyperalgesia have been described already. For example, increased intracellular calcium results in desensitization of TRPV1 (Caterina *et al.* 1997; Numazaki *et al.* 2003). Also, desensitization is the result of direct phosphorylation of TRPV1 by CaMKII, a kinase activated by elevated calcium levels (Price *et al.* 2005). Accordingly, lowering of intracellular calcium concentrations has been correlated with increased spontaneous activity of nociceptive neurons (Fuchs *et al.* 2005; Hogan *et al.* 2008). Surprisingly, not only the increase but also the block of CaMKII activity has been reported to ablate hyperalgesia (Fang *et al.* 2002; Chen *et al.* 2010; Liang *et al.* 2011; Matsumura *et al.* 2011). Some of these seemingly contradictory results might be attributed to differential effects of CaMKII if activated in the peripheral versus central nervous system. On the basis of our results, we suggest an alternative explanation. CaMKII appears to be not only involved in the direct action on effector ion channels, such as TRPV1. Instead, it also regulates the sensitization signaling network, which is involved in the computation of sensitizing inputs and which is initiated mainly by GPCRs.

Extracellular mediator-dependent nociceptor sensitization has been widely reported. The response of the nociceptive neuron to a physical stimulus like pressure or temperature is altered if the environmental context is altered by e.g., an ongoing inflammation. We now tested for an additional aspect of context dependence. We investigated if not only the environmental context but also the intracellular signaling context itself results in modulation of sensitization. Information concerning this is important, as in physiological conditions like inflammation, more than one GPCR is activated. It is also important, as one is tempted to assume that one can simply combine results of the common one-stimulus approaches to explain the outcome of multiple-stimuli experiments.

One central conclusion of our study is that the result of the combination of sensitizing stimuli cannot be deduced simply from responses of sensitizing agents, which were applied individually. We show that known sensitizing stimuli such as isoproterenol, ESCA, and estrogen receptor GPR30-specific G1 can be converted to antinociceptive signaling depending on the signaling context (see Fig. 6 for overview). Apparently, the information, if a stimulus is sensitizing or desensitizing, is not encoded in the extracellular mediator itself. Instead, the cell has far-reaching abilities to 'interpret' the incoming information. In our experiments, the

outcome of such 'interpretation' goes even to the extreme that a 'sensitizing' stimulus results in desensitization. This is very much in agreement with results obtained with a macrophage cell line, where the combination of two stimuli resulted in synergistic but also antagonistic effects in an often unexpected, but defined and context-dependent manner (Natarajan *et al.* 2006). Thus, the current differentiation of extracellular mediators into sensitizing and non-sensitizing has to be taken with caution. The stimulus context is apparently of considerable significance for the cellular nociceptive response.

The dependence of sensitizing mediators on the stimulus context is a great challenge. The outcome of various combinations of well-known "sensitizing" stimuli such as PGE2, 5HT, growth factors, Bradykinin, and others has to be established in experiments with two and more stimuli. But with any additional stimulus, the degree of freedom and thus the number of control experiments to be performed increases considerably. Not only the stimuli, but also the order of all stimuli, including the variation of time intervals between them have to be tested. Nevertheless, the possibility of non-additivity and even inversion of sensitizing stimuli illustrates the need to better understand pain sensitization in more complex, i.e., more physiological context.

We found sensitization signaling to be blocked quickly and long lastingly. One important aspect, which requires further investigation, is to analyze, which mechanism maintains this long-term inhibition. Bistable positive forward loops of kinases were shown to accomplish such stable context memory e.g., in Xenopus eggs (Xiong and Ferrell 2003). One kinase activated by increases in calcium is CaMKII. This kinase was found to be essential for the establishment of long-term changes in the CNS, such as long-term potentiation. A recent report indicates that the pharmacogenetic over-activation of CaMKII results in memory extinction in the CNS (Cao *et al.* 2008). In accordance with a positive forward loop underlying cellular-context memory proposed by Ferrel *et al.*, CaMKII can long lastingly increase the intracellular calcium concentration (Curran *et al.* 2007). Indeed, one might take calcium traces such as shown in Fig. 3a as a first indication for such an increased homeostatic level of cytoplasmic calcium, although this has to be investigated in detail in future works.

Calcium homeostasis is central to nociception. Calcium concentration in peripheral sensory neurons in models of experimental neuropathic pain has been shown to be lowered (Fuchs *et al.* 2005). Hogan *et al.* found that an increase of the constitutively lowered calcium concentration in DRG neurons reverses hyperexcitability (Hogan *et al.* 2008). Interestingly, they also found hyperexcitability to correlate with reduced CaMKII responsiveness (Kawano *et al.* 2009). Our findings are complementary to theirs, showing that an increase of cytoplasmic calcium results in a block of future sensitizing pathways as well as reversal of established hyperalgesia. Thus, Hogan and our results suggest that some mechanisms central to neuropathic and inflammatory pain might be shared and are potentially sites for intervention.

CaMKII and Epac play an important role in long-term potentiation (Lisman *et al.* 2002; Gekel and Neher 2008). Thus, it will be interesting to investigate, if similar signal inversions exist in the CNS mediated by a similar context dependence as shown for the peripheral nociceptive neuron. This is of special interest as we have shown desensitization through *activation* of *endogenous* signaling pathways. Thereby, we extend mechanisms for loss of memory which have been described in the CNS to occur following lack of reconsolidation (Debiec *et al.* 2006), pharmacological inhibition of maintenance mechanisms (Ling *et al.* 2002; Shema *et al.* 2007), or after pharmacogenetic change of CaMKII activity (Cao *et al.* 2008).

In conclusion, we have characterized a counterintuitive aspect of cellular signal integration. We have shown that sensitizing signaling can have a desensitization signaling branch. This branch not only blocks but inverts a sensitization signaling pathway resulting in desensitization. This inversion was not dependent on protein expression as in other examples of context dependence, but on the signaling activity in nociceptive neurons (Hucho *et al.* 2005; Rush *et al.* 2006). Our results thereby indicate that peripheral pain can be attenuated by the *activation* (sic) and *rerouting* (sic) of *endogenous* signaling cascades, which could decouple e.g., inflammatory processes important for tissue regeneration from pain sensitization. This could be of therapeutic importance for the therapy of ongoing pain as well as preemptive treatment.

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