Fast Ca$^{2+}$-Induced Potentiation of Heat-Activated Ionic Currents Requires cAMP/PKA Signaling and Functional AKAP Anchoring

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Distler, C., P. K. Rathee, K. S. Lips, O. Obreja, W. Neuhuber, and M. Kress Fast Ca$^{2+}$-induced potentiation of heat-activated ionic currents requires cAMP/PKA signaling and functional AKAP anchoring. J Neurophysiol 89: 2499–2505, 2003; 10.1152/jn.00713.2002. Calcium influx and the resulting increase in intracellular calcium concentration ([Ca$^{2+}$]$_i$) can induce enhanced sensitivity to temperature increases in nociceptive neurons. This sensitization accounts for heat hyperalgesia that is regularly observed following the activation of excitatory inward currents by pain-producing mediators. Here we show that rat sensory neurons express calcium-dependent adenylyl cyclases (AC) using RT-PCR and nonradioactive in situ hybridization. Ionomycin-induced rises in [Ca$^{2+}$]$_i$-activated calcium-dependent AC and caused translocation of catalytic protein kinase A subunit. Elevation of [Ca$^{2+}$]$_i$ finally resulted in a significant potentiation of heat-activated currents and a drop in heat threshold. This was not prevented in the presence of suramin that nonspecifically uncouples G protein-dependent receptors. The sensitization was, however, inhibited when the specific PKA antagonist PKI$_{14-22}$ was added to the pipette solution or when PKA coupling to A kinase anchoring protein (AKAP) was disrupted with InCELLect StHt-31 uncoupling peptide. The results show that heat sensitization in nociceptive neurons can be induced by increases in [Ca$^{2+}$]$_i$, and requires PKA that is functionally coupled to the heat transducer, mostly likely vanilloid receptor VR-1. This calcium-dependent pathway can account for the sensitizing properties of many excitatory mediators that activate cationic membrane currents.

INTRODUCTION

Excitatory chemical mediators like ATP, acetylcholine, or extracellular acidosis not only cause burning pain but also increase nociceptor responsiveness to cause heat hyperalgesia (Belmonte et al. 1991; Bernardini et al. 2001; Burnstock and Wood 1996; Guenther et al. 1999; Kress and Guenther 1999; Steen et al. 1992). Similarly, the pungent ingredient of red hot chili peppers, capsaicin, sensitizes nociceptive afferents to heat and leaves the application site in a hypersensitive state (LaMotte et al. 1992; Schmelz and Kress 1996; Simone et al. 1987). The common principle of action of these substances is that they activate ionic currents that cause calcium influx and consecutive rises in intracellular calcium concentration ([Ca$^{2+}$]$_i$) (Bevan and Yeats 1991; Bouvier et al. 1990; Garcia-Hirschfeld et al. 1995; Oh et al. 1996; Zeilhofer et al. 1996, 1997). A number of receptor ion channel complexes have been cloned that, when activated, are permeable to calcium ions and expressed in rat sensory neurons (Caterina et al. 1997; Chen et al. 1995; Genzen et al. 2001; Gray et al. 1996; Hayes et al. 2000; Tominaga et al. 1998). Rises in [Ca$^{2+}$]$_i$, account for the heat sensitization induced by the excitatory agents ATP, acidic pH, capsaicin, and a number of experimental compounds (Guenther et al. 1999; Kress and Guenther 1999). However, the downstream signaling cascade of the sensitization process is unknown at the cellular level. Among calcium-triggered signaling cascades, PKC activation can facilitate heat transduction via vanilloid receptor-1 (VR-1) modification (Cesare et al. 1999; Chuang et al. 2001; Premkumar and Ahern 2000; Tominaga et al. 2001). Despite the observation that the capsaicin analogue resiniferatoxin activates PKC, this enzyme is not activated by the other mediators (Harvey et al. 1995). Alternatively, heat-activated ion channels like VR-1 can be affected by the cAMP/PKA cascade (De Petrocellis et al. 2001). Proinflammatory PGE$_2$ induced heat sensitization of sensory neurons by activating receptor subtypes (EP3C and EP4) that are coupled to the cAMP/PKA cascade and sensitization to heat also occurred in the presence of membrane-permeant cAMP analogues activating PKA (Kress et al. 1996; Kumazawa et al. 1996; Southall and Vasko 2001). Furthermore mice carrying a null mutation for type 1B PKA regulatory subunit no longer exhibited increased heat pain behavior following PGE$_2$ administration (Malberg et al. 1997). In a cellular model, capsaicin-activated ion currents became facilitated in the presence of adenyl cyclase activator forskolin (Lopshire and Nicol 1998) and VR-1 phosphorylation at PKA consensus sites potentiated heat-induced currents (Rathee et al. 2002). In the present study we investigate whether the cAMP/PKA signaling cascade is involved in the potentiation of heat-activated ionic currents by rises in [Ca$^{2+}$]$_i$ in a cellular model.

METHODS

Preparation of neuronal cultures

A detailed description of the dissociation has been published elsewhere (Guenther et al. 1999). Briefly, lumbar dorsal root ganglia (DRG, L1–L5) were harvested from adult female Wistar rats weighing 110 to 160 g from an inbred colony. The connective tissue was removed and the ganglia were treated with collagenase (0.28 U/ml, Roche Biochemicals, Mannheim Germany) for 75 min and trypsin...
(25,000 U/ml in PBS, PAA Laboratories, Coelbe, Germany) for 12 min in a humid atmosphere containing 5% CO₂ at 37°C. The cells were dissociated with a fire-polished Pasteur pipette, plated on poly-L-lysine coated (200 μg/ml Sigma) coverslips and cultivated in serum-free TNB 100 medium (Biochrom, Berlin) supplemented with penicillin-streptomycin (each 20,000 IU/100 ml), 2 mM L-glutamine (both from GIBCO), and 100 ng/ml NGF (mouse NGF 7S, 100 ng/ml, Alomone Labs, Tel Aviv, Israel).

Electrophysiology

Whole cell current measurements in the voltage-clamp configuration of the patch-clamp technique were performed ≤40 h after dissociation at ~80 mV holding potential and 3 kHz sampling rate with an Axopatch 200A amplifier and pClamp6.0 software package on a PC-type computer (Axon Instruments, Forster City, CA). Small-size capsaicin-sensitive neurons were selected, and only those neurons that exhibited heat-activated currents in response to the standard heat stimuli used were processed because they are generally assumed to represent polymodal nociceptors. Borosilicate glass electrodes (Science Products, Hofheim, Germany) pulled on a horizontal puller (Sutter Instrument Company, Novato, CA) had resistances of 2–5 MΩ after filling with (in mM) 148 KCl, 4 MgCl₂, 2 Na-ATP, 10 HEPES, and 0.2 Li-GTP, with the pH adjusted to 7.3 with KOH. The external solution consisted of (in mM) 145 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES, at pH 7.3 adjusted with NaOH.

Ratioometric Ca²⁺ measurements

FURA-2 (100 μM, pentapotassium salt, Molecular Probes, Leiden, The Netherlands) was added to the intracellular solution and passively loaded into the cell via the patch pipette (Gryniewicz et al. 1985). Background corrected fluorescence images were taken with a slow-scan CCD camera system with a fast monochromator (PTI) coupled to an Axiovert with a 40× fluor oil immersion objective (Zeiss, Oberkochen, Germany). FURA-2 was excited at 340 and 380 nm wavelengths with equal exposure times of 200 ms and fluorescence was collected at λ > 500 nm [Ca²⁺], was calculated as previously published (Gryniewicz et al. 1985; Kress and Guenther 1999).

Heat and chemical stimulation

For drug application and heat stimulation of single neurons, a fast seven-channel system with common outlet was used, which allowed for independent heating of all seven solutions. Magnetic valves to control opening and close the reservoirs were controlled manually from a switchboard and voltage commands for automated heat stimulation were performed in presence of 1% normal goat serum and human immune globulin in PBS (pH 7.5). Sections were washed in standard sodium citrate buffer (20 min 2x buffer and 20 min 1x buffer), 20 μg/ml RNase A (Sigma, 30 min, 37°C), decreasing concentrations of 1x, 0.5x, 0.2x standard citrate buffer (1x and 0.5x for 20 min and 0.2x for 20 min, 1 h 50°C, 20 min 24°C), distilled water (5 min), 0.1 M Tris-HCl (10 min) and 0.1 M maleate buffer (10 min). Detection of the DIG-labeled probe was performed as recommended by the manufacturer, with alkaline phosphatase conjugated DIG-antibody (4°C, 12 h). Color development was allowed to proceed in the dark for 4–16 h. The reaction was terminated by immersion in PBS (pH 7.5). Sections were mounted with glycerol jelly (Merck, Darmstadt, Germany).

Indirect immunocytochemistry

For detecting vanilloid receptor 1 (VR-1) and protein kinase A (PKA) subunits indirect immune fluorescence was performed using primary monoclonal IgG immune sera anti-PKA-RI, anti-PKA-RII and anti-PKA-C (BD Transduction Labs, Franklin Lakes, USA) applied in presence of 10% fetal bovine serum, 0.5% Triton X-100 (TX), 1% normal goat serum (NGS), and human immune globulin (Cohn’s fraction II, 2 mg/ml Sigma) in phosphate buffered saline (PBS) for 24 h at 4°C. Secondary antibodies coupled to Alexa 488 (Molecular Probes) or Cy3 (Dianova, Hamburg, Germany) were applied in presence of 1% normal goat serum and human immune globulin in PBS for 60 min at room temperature. Coverslips were mounted on glass slides with glycerol jelly and were analyzed with confocal laser scanning microscopy (Biorad MRC 1000 attached to a Nikon Diaphot 300). Excitation of the Alexa 488 was performed with the 488 nm line of a Krypton-Argon mixed gas laser (Ion Laser Technology, Salt Lake City). Single confocal optical sections were obtained with a 60x oil immersion objective (N.A. 1.4). Confocal images were converted using the program “Confocal Assistant 4.02” Build 101 1994–1996 by Todd Clark Brejl. The length/profile function of COMOS software (Biorad) was used to quantify peripheral translocation of PKA immune staining. The total average fluorescence intensity over the cell diameter (F) was calculated and set to 1. To quantify the redistribution of PKA catalytic subunit, average fluorescence intensities were calculated for 10% segments of the total intensity profile length (AF),
normalized to F (ΔF/F) and compared between peripheral (P) and central regions of the cell (C).

Data analysis

For detailed statistical analysis the Statistica software package for Windows 6.0 (StatSoft, Tulsa, OK) was used. All summarizing results are given as means ± SE. For intraindividual data comparisons, the Wilcoxon matched pairs test was calculated, if not stated otherwise. For interindividually comparisons of independent groups the Mann-Whitney U test was used. Differences were considered significant at P < 0.05.

RESULTS

Involvement of protein kinase A (PKA) in Ca2+-induced potentiation of Iheat in sensory neurons

Only neurons sensitive to the nociceptor excitant capsaicin (1 μM) were included in the study. In these neurons, short exposure to ionomycin (5 s) resulted in a rise in [Ca2+]i, from 107 ± 24 nM to 366 ± 45 nM (n = 8) and recovered within 5 min. Heat-activated inward currents were significantly potentiated from 608 ± 141 pA to 979 ± 220 pA (P < 0.05, n = 8; Fig. 1) following the elevation of [Ca2+]i, and this sensitization of Iheat was fully reversible as published previously for step shaped 2 s heat stimuli (Guenther et al. 1999; Kress and Guenther 1999). To determine activation thresholds of Iheat ramp shaped heat stimuli were used in the present study and a drop of heat activation thresholds from 45.1 ± 0.5°C to 42.6 ± 0.7°C was observed following the increase [Ca2+]i (average drop 2.4 ± 0.6°C, P < 0.05, n = 8, see Fig. 3).

Involvement of protein kinase A (PKA) in Ca2+-induced sensitization of Iheat

Since rises in intracellular calcium levels have been linked to the activation of the cAMP/PKA signaling cascade we investigated if the calcium-induced potentiation of Iheat was dependent on PKA activation. For this purpose, the selective PKA inhibitor PKI14–22 was added to the pipette solution and equilibrated with the cytoplasm of the recorded cells for ≥5 min after establishing the whole cell configuration. Under these conditions, ionomycin-induced rises in [Ca2+]i, were unaltered (118 ± 16 nM before to 391 ± 64 nM after ionomycin). However, the potentiation of heat responses was almost completely prevented (476 ± 74 pA before versus 510 ± 85 pA after ionomycin; n = 8, P < 0.05; Fig. 2). In addition, no shift of heat thresholds toward lower temperatures was observed (44.2 ± 1.3°C before vs. 43.8 ± 1.2°C after ionomycin; Fig. 3). More support for a calcium-mediated activation of the cAMP/PKA cascade was obtained from indirect immunocytochemistry and confocal image analysis of PKA expression in isolated DRG neurons. While PKA regulatory subunit expression was detected in the vicinity of the plasma membrane, PKA catalytic subunit (PKA-C) was evenly distributed throughout the cytoplasm in control cells (Rathee et al. 2002). In the present study, PKA-C translocated to the cell periphery fol-

FIG. 1. Ionomycin induced potentiation of heat-activated ionic currents (Iheat). A: example of a DRG neuron that was repetitively exposed to noxious heat before and after ionomycin application (1 μM, 5 s) and intracellular calcium concentration (bottom). B: mean peak heat responses of 8 neurons and corresponding rises in intracellular calcium concentration (lower panel). Asterisks mark significant differences (P < 0.05). Application of ionomycin (5 s) did not itself induce inward or outward currents at room temperature.

FIG. 2. Inhibition of PKA prevents Ca2+-induced potentiation of Iheat. A: example of a neuron that responded to ionomycin with a clear rise in intracellular calcium concentration (bottom) but did not exhibit potentiation of Iheat when the selective PKA inhibitor peptide PKI14–22 was added to the pipette solution (top). B: summary of heat-activated currents and ionomycin-induced rises in [Ca2+]i of 8 cells in the presence of PKI14–22.

FIG. 3. Inhibition of PKA prevents Ca2+-induced drop in heat threshold. A: drop in heat threshold before and after ionomycin application as observed in a single neuron (top) and summary of changes in 8 cells (bottom) B: no changes of heat threshold were observed as depicted for a representative example (top) or on the average (bottom) when the selective PKA inhibitor PKI14–22 was added to the pipette solution.
lowing exposure to ionomycin. This was quantified for 10/10 neurons using line profile analysis of fluorescence intensities, and examples of staining as well as average data on-line profile analysis are given in Fig. 4.

Expression of Ca\(^{2+}\)-dependent adenylyl cyclases in DRG neurons

Since the sensitizing effect of intracellular calcium rises was almost immediate we proposed the contribution of calcium activated enzymes as a potential mechanism and looked for expression of calcium dependent adenylyl cyclases (AC). RT-PCR revealed expression of mRNA for the Ca\(^{2+}\) dependent AC I and AC VIII in 98% of VR-1 positive and negative rat lumbar DRGs (Fig. 5A). To determine if the calcium dependent ACs were localized in neurons or in non-neuronal cells, in situ hybridization with gene-specific antisense and sense probes was performed on cryosections of rat DRG. AC I as well as AC VIII mRNA was located in the majority of neurons including medium and small diameter nociceptive neurons with almost an even distribution throughout the cytoplasm (Fig. 5B). All other cell types in the DRG sections, i.e., satellite cells, Schwann cells, endothelial cells and the ganglionic capsular cells were negative. No signal was detected in sections treated with the sense probes as negative controls. To address the possibility of calcium-dependent prostanoid synthesis and autacoid activation of Gs protein-coupled prostanoid receptors with consecutive AC/PKA activation some experiments were performed in the presence of suramin (10\(^{-4}\) M) to non-specifically uncouple G proteins from such receptors (Freissmuth et al. 1999). Under these conditions a similar increase in [Ca\(^{2+}\)]i from 152 ± 26 nM to 527 ± 83 nM and a similar potentiation of \(I_{\text{heat}}\) from 460 ± 125 pA to 755 ± 205 pA (\(n = 5\), \(P < 0.05\)) was observed after exposure to ionomycin as compared with controls. Also, the shift in heat thresholds was preserved (42.5 ± 0.3°C before vs. 40.4 ± 0.3°C after ionomycin, \(n = 5\), \(P < 0.5\) Fig. 6).

Heat sensitization involves PKA anchoring via an AKAP

In many cell types localization and targeting of PKA is mediated by spatial interaction with A kinase anchoring proteins (AKAPs). To investigate the functional importance of these anchoring proteins in calcium-induced heat sensitization, experiments were performed in the presence of the InCELLect\textsuperscript{®} AKAP St-Ht31 inhibitor peptide (10\(^{-5}\) M) which disrupts PKA interaction with AKAP. Addition of the peptide to the intracellular solution and equilibration with the cell cytoplasm for ≥5 min after establishing the whole cell configuration did not alter ionomycin-induced rises in [Ca\(^{2+}\)]i (from 70 ± 12 to 986 ± 358 nM, \(n = 8\), \(P < 0.05\)). However, in the presence of the peptide, increases in heat responses were no longer observed (535 ± 81 pA before versus 528 ± 98 pA after ionomycin; Fig. 7). Addition of the InCELLect AKAP St-Ht31-P negative control peptide to the pipette solution did not

**FIG. 4.** PKA catalytic subunit translocates to the cell periphery after ionomycin stimulation. A: confocal images of control and ionomycin stimulated neurons stained for catalytic subunit of PKA (PKA-C). Bar = 10 \(\mu\)m. The positions of the line scan profile used for calculating the fluorescence profiles given in B for the same cells are indicated. C: average fluorescence magnitude in peripheral (P) and central (C) regions of the cell divided by the total average fluorescence of the cell exhibiting translocation of PKA-C to the cell periphery after ionomycin.

**FIG. 5.** RT-PCR and in situ hybridization reveal expression of calcium-dependent adenylyl cyclases. A: single bands of appropriate size (400 bp, ACVIII, ACI and PKA-R1, RII; 500 bp, PKA-C) and sequence were detected in lumbar DRGs. B: antisense riboprobes for ACI (A, C) and ACVIII (D, F) labeled the majority of DRG neurons including medium and small diameter nociceptive neurons. In higher magnification (C, F) the distribution throughout the neuronal cytoplasm is demonstrated. The nonneuronal cells were negative. (B, E) Negative controls with sense riboprobes. Bar = 100 \(\mu\)m in A-B, D-E, and 50 \(\mu\)m in C, F.
Calcium-induced potentiation of $I_{\text{heat}}$ mediated by PKA

**DISCUSSION**

The present study investigates the downstream signaling pathway activated by rises in intracellular calcium concentration $[Ca^{2+}]_i$, that cause heat sensitization of rat primary afferent nociceptors. Evidence is presented that calcium-dependent adenylyl cyclases (ACs) are expressed in sensory neurons together with PKA subunits and that calcium-induced potentiation of $I_{\text{heat}}$ under control conditions or in the presence of suramin; $n = 5$. $B$: the calcium-induced drop of heat thresholds was preserved in the presence of suramin in single neurons (top) or on the average (bottom).

Calcium ions can induce the activation of phospholipase A2 and consecutive production and autacoid activation of prostanoíd receptors (Murakami et al. 1999; Southall and Vasko 2001). However, the nonselective G protein inhibitor suramin (Butler et al. 1988; Freissmuth et al. 1999) did not affect the calcium-induced potentiation of $I_{\text{heat}}$. Therefore indirect effects via autacoid activation of a G protein-coupled receptors are unlikely to contribute to downstream signaling and potentiation of $I_{\text{heat}}$.

Among the enzymes activated by increases in $[Ca^{2+}]_i$, G protein-independent adenylyl cyclases type I and VIII have been found in mouse embryonic DRG neurons (Fields et al. 1997). In the present study, RT-PCR and in situ hybridization assays detected expression of these ACs in the majority of small and medium size DRG neurons from adult rat. Furthermore, ionomycin induced translocation of PKA catalytic subunit and, in ionic current recordings, the ionomycin-induced potentiation of $I_{\text{heat}}$ was completely abolished during selective PKA inhibition. This strongly argues for a calcium-induced activation of the AC/PKA cascade. Although calcium-mediated cGMP increase has occurred in sensory neurons, a major role of this nucleotide in the sensitization process presented here is unlikely since cGMP did not affect nociceptor heat sensitivity (Kress et al. 1996; Wood et al. 1989).

As a potential target of PKA phosphorylation the vanilloid receptor VR-1 was suggested for two reasons: first, capsaicin-activated ionic currents were potentiated after FSK stimulation (Lopshire and Nicol 1998) and, second, in mice carrying a null mutation for VR-1 thermal hyperalgesia following inflammation was greatly reduced (Caterina et al. 2000; Davis et al. 2000). More hints toward the relevance of PKA phosphorylation of VR-1 also came recently from a biochemical study and from a cellular study showing that AC/PKA mediated potentiation of VR-1 heat responses (De Petrocellis et al. 2001; Rathee et al. 2002). It is suggested that the calcium-dependent activation of cAMP/PKA causes a similar potentiation of VR-1 since the potentiation was prevented in the presence of selective PKA inhibitor in the present study.

Calcium-induced heat sensitization also depended on functional coupling of heat transducing ion channel via an AKAP as known for other targets of PKA phosphorylation in many cell types including neurons (Hayabuchi et al. 2001; Klussmann et al. 1999; Potet et al. 2001; Rosenmund et al. 1994; Xie and Raufman 2001). Regulatory and catalytic PKA subunits as well as a number of AKAPs are co-expressed in sensory neurons (Rathee et al. 2002). Most of the AKAPs identified so far preferentially bind RII subunit and some of the AKAPs, e.g., Yotiao or AKAP 15/18, directly target PKA to ion channels (Colledge and Scott 1999; Johnson et al. 1994). Two AKAPs have been identified that exhibit dual specificity binding to RI as well as RII subunits, e.g., AKAP-KL or dAKAPs 1 and 2 and the dual dAKAP-2 has been found in DRG neurons (Colledge and Scott 1999; Huang et al. 1997a,b; Rathee et al. 2002). Members of the dual AKAP subfamily may be the appropriate candidates for coupling PKA to VR-1.

**FIG. 6.** $Ca^{2+}$-induced potentiation of $I_{\text{heat}}$ is preserved in the presence of suramin. $A$: significant increase in intracellular calcium concentration and potentiation of $I_{\text{heat}}$ under control conditions or in the presence of suramin; $n = 5$. $B$: the calcium-induced drop of heat thresholds was preserved in the presence of suramin in single neurons (top) or on the average (bottom).

**FIG. 7.** $Ca^{2+}$-induced potentiation of $I_{\text{heat}}$ is prevented in the presence of specific A kinase anchoring protein inhibitor. InCELLect AKAP St-H31 prevents the $Ca^{2+}$-induced potentiation of heat-activated currents. Ionomycin-induced rises in $[Ca^{2+}]_i$ are not affected.
for phosphorylation of the channel and potentiation of the heat responses. In the present study, ionomycin did not induce heat sensitization in the presence of the AKAP uncoupling peptide InCELLect St-Hi31 which again strongly argues for an involvement of PKA and functional coupling of the enzyme to the ion channel via an AKAP in the present mechanism. In contrast to PKC which is considered a classical translocation enzyme involved in heat sensitization translocation of PKA catalytic subunits to the cell periphery is a relatively new finding (Cesare et al. 1999; Rathee et al. 2002). The present study reveals a translocation of PKA catalytic subunit toward the cell periphery on exposure to ionomycin which further supports a calcium-triggered PKA activation and consecutive phosphorylation of a membrane bound target protein e.g., VR-1. However, contribution of other heat-activated ion channels of the TRP V family, e.g., TRP V-3 or TRPV-4 at present cannot be excluded (Peier et al. 2002; Güler et al. 2002).

In summary, we demonstrate that in nociceptive neurons rises in [Ca^{2+}]_{i} induced a transient and reversible translocation of PKA catalytic subunit to the cell periphery and a potentiation of I_{heat} which was abolished after inhibition of PKA. In addition, the calcium-induced potentiation of I_{heat} depended on functional anchoring of PKA via AKAPs. Our results provide evidence for a calcium-triggered potentiation of heat-activated ionic currents, possibly VR-1 or others, by PKA phosphorylation. This mechanism may link excitatory mediators like ATP, nicotine or acidic pH to heat hyperalgesia following inflammation or cell death.

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