BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2012.02174.x
www.brjpharmacol.org

RESEARCH PAPER

AKAP-dependent sensitization of $Ca_v3.2$ channels via the EP_4 receptor/cAMP pathway mediates PGE_2-induced mechanical hyperalgesia

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Keywords
$Ca_v3.2$ T-type calcium channel; mechanical hyperalgesia; PGE_2; EP_4 receptor; PKA; A-kinase anchoring protein 150 (AKAP150)

Received
10 December 2011
Revised
30 July 2012
Accepted
15 August 2012

BACKGROUND AND PURPOSE

The $Ca_v3.2$ isoform of T-type Ca^{2+} channels (T channels) is sensitized by hydrogen sulfide, a pro-nociceptive gasotransmitter, and also by PKA that mediates PGE_2-induced hyperalgesia. Here we examined and analysed $Ca_v3.2$ sensitization via the PGE_2/cAMP pathway in NG108-15 cells that express $Ca_v3.2$ and produce cAMP in response to PGE_2, and its impact on mechanical nociceptive processing in rats.

EXPERIMENTAL APPROACH

In NG108-15 cells and rat dorsal root ganglion (DRG) neurons, T-channel-dependent currents (T currents) were measured with the whole-cell patch-clamp technique. The molecular interaction of $Ca_v3.2$ with A-kinase anchoring protein 150 (AKAP150) and its phosphorylation were analysed by immunoprecipitation/immunoblotting in NG108-15 cells. Mechanical nociceptive threshold was determined by the paw pressure test in rats.

KEY RESULTS

In NG108-15 cells and/or rat DRG neurons, dibutyryl cAMP (db-cAMP) or PGE_2 increased T currents, an effect blocked by AKAP St-Ht31 inhibitor peptide (AKAPI) or KT5720, a PKA inhibitor. The effect of PGE_2 was abolished by RQ-00015986-00, an EP_4 receptor antagonist. AKAP150 was co-immunoprecipitated with $Ca_v3.2$, regardless of stimulation with db-cAMP, and $Ca_v3.2$ was phosphorylated by db-cAMP or PGE_2. In rats, intraplantar (i.pl.) administration of db-cAMP or PGE_2 caused mechanical hyperalgesia, an effect suppressed by AKAPI, two distinct T-channel blockers, NNC 55-0396 and ethosuximide, or $ZnCl_2$, known to inhibit $Ca_v3.2$ among T channels. Oral administration of RQ-00015986-00 suppressed the PGE_2-induced mechanical hyperalgesia.

CONCLUSION AND IMPLICATIONS

Our findings suggest that PGE_2 causes AKAP-dependent phosphorylation and sensitization of $Ca_v3.2$ through the EP_4 receptor/cAMP/PKA pathway, leading to mechanical hyperalgesia in rats.

Abbreviations

AKAP, A-kinase anchoring protein; AKAPI, AKAP St-Ht31 inhibitor peptide; AUC, area under the curve; db-cAMP, dibutyryl cyclic AMP; DRG, dorsal root ganglion; HVA, high voltage-activated; i.pl., intraplantar; N-channel, N-type calcium channel; T channel, T-type calcium channel; T current, T-channel-dependent current; TRPV1, transient receptor potential vanilloid-1

Introduction

PGE_2, a mediator for inflammatory pain, causes sensitization of the primary nociceptive neurons (nociceptors). There is plenty of evidence that increased cAMP levels followed by activation of PKA mediate the peripheral PGE_2-induced sensitization of nociceptors and subsequent hyperalgesia, although PKC may also be involved in the peripheral pronociceptive effect of PGE_2 (Moriyama *et al.*, 2005; Sachs *et al.*, 2009; Kawabata, 2011). Among four subtypes of PGE_2 receptors, EP_2 and EP_4 receptors are coupled to G_S protein and, when activated, stimulate the cAMP/PKA pathway; while stimulation of EP_1 receptors, coupled to Gq protein, causes activation of PKC accompanied by cytosolic Ca^{2+} mobilization (Kawabata, 2011). Increasing evidence demonstrates that PGE_2-induced activation of the EP_4/PKA and EP_1/PKC cascades in the peripheral nociceptors plays major roles in the development of various types of inflammatory pain and/or hyperalgesia (Moriyama *et al.*, 2005; Lin *et al.*, 2006; Nakao *et al.*, 2007; Clark *et al.*, 2008; Murase *et al.*, 2008a; Colucci *et al.*, 2010; Miki *et al.*, 2011). In nociceptors, PKA and PKC phosphorylate and sensitize transient receptor potential vanilloid-1 (TRPV1) channels in a manner dependent on A-kinase anchoring protein 150 (AKAP150) and AKAP79, the rodent and human homologues of a scaffold protein respectively (Moriyama *et al.*, 2005; Zhang *et al.*, 2008). Thus, sensitization of TRPV1, a sensor of noxious heat, via the EP_4/PKA and EP_1/PKC pathways appears to mediate the PGE_2-evoked thermal hyperalgesia. Nonetheless, mechanical hyperalgesia caused by PGE_2 may not be attributable to sensitization of TRPV1 but involve other mechanisms. Thus, molecules that mediate the PGE_2-induced mechanical hyperalgesia have yet to be identified.

Low voltage-gated (T-type) Ca^{2+} channels (T channels), particularly of the $Ca_v3.2$ isoform, are abundantly expressed in nociceptors and involved in peripheral nociceptive processing (Todorovic *et al.*, 2001; Nelson *et al.*, 2007; Todorovic and Jevtovic-Todorovic, 2011). We have shown that $Ca_v3.2$ is targeted by hydrogen sulfide, an endogenous gasotransmitter, and plays a critical role in inflammatory and/or neuropathic pain including visceral pain (Kawabata *et al.*, 2007; Maeda *et al.*, 2009; Matsunami *et al.*, 2009; 2011; Nishimura *et al.*, 2009; Takahashi *et al.*, 2010). Most interestingly, T channels including $Ca_v3.2$ can be phosphorylated and sensitized by PKA (Kim *et al.*, 2006; Chemin *et al.*, 2007), although conflicting evidence for PKC modulation of T channels has been reported (Park *et al.*, 2006; Chemin *et al.*, 2007; Rangel *et al.*, 2010; Zhang *et al.*, 2011). In this context, we hypothesize that sensitization of $Ca_v3.2$ T channels by PKA might contribute to PGE_2-induced mechanical hyperalgesia.

In the present study, using NG108-15 cells (mouse neuroblastoma × rat glioma hybrid cells) in which $Ca_v3.2$ is abundantly expressed and PGE_2 increases cAMP production (Gylys *et al.*, 1997; Chemin *et al.*, 2002; Nagasawa *et al.*, 2009; Tarui *et al.*, 2010), we thus asked if PGE_2 is capable of sensitizing $Ca_v3.2$ T channels and analysed the underlying molecular mechanisms including the possible involvement of EP_4 receptors and AKAP150. To our knowledge, there has been no study indicating the relationship between EP receptors and AKAPs, whereas AKAP79/150 has been shown to anchor β-adrenoceptors, PKA and adenylyl cyclase (Welch *et al.*, 2010). Since EP_4, like β-adrenoceptors, is a Gs-coupled receptor it is likely that AKAPs might also function as a scaffolding protein forming the downstream signalling complexes of EP_4 receptors. Furthermore, we also determined whether the EP_4/PKA/T-channel pathway contributes to the development of mechanical hyperalgesia following intraplantar (i.pl.) administration of PGE_2 in rats.

Methods

Chemicals

Dibutyryl cyclic AMP (db-cAMP), PGE_2, NNC 55-0396, ethosuximide, $ZnCl_2$, verapamil, IBMX, SB366791 and FK506 were purchased from Sigma-Aldrich (St. Louis, MO, USA). AKAP St-Ht31 inhibitor peptide (AKAPI) was obtained from Promega Corporation (Madison, WI, USA). ω-Conotoxin GVIA and ω-conotoxin MVIIC were purchased from Peptide Institute, Inc. (Osaka, Japan). KT5720 was from Cayman Chem. (Ann Arbor, MI, USA). Nifedipine was from Wako Pure Chem. (Osaka, Japan). RQ-00015986-00 (CJ-042794) was kindly donated by RaQualia Pharma Inc. (Aichi, Japan). NNC 55-0396, db-cAMP, ethosuximide, $ZnCl_2$, verapamil, ω-conotoxin GVIA, ω-conotoxin MVIIC and AKAPI were dissolved in distilled water or saline. Nifedipine and PGE_2 were dissolved in ethanol (Wako Pure Chem.); and RQ-00015986-00, FK506 and KT5720 were dissolved in DMSO (Sigma-Aldrich), for the experiments with NG108-15 cells (the final concentrations of ethanol and DMSO, 0.1%). For the experiments using rats, PGE_2 was dissolved in ethanol then diluted with saline (the final ethanol concentration, 0.0284%). RQ-00015986-00 was suspended in 0.1% methyl cellulose 400 (Wako Pure Chem.) for oral administration to rats. SB366791 was suspended in the mixture of 2% DMSO, 1% Cremophor® EL (Nacalai Tesque, Inc., Kyoto, Japan) and 97% saline.

Experimental animals

Male Wistar rats (5 weeks old) were purchased from Kiwa Laboratory Animals Co., Ltd. (Wakayama, Japan) and used for the experiments at the age of 6–8 weeks. A total of 280 rats were used. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). All animals were used with approval by the Committee for the Care and Use of Laboratory Animals at Kinki University, and all procedures employed in the present study were in accordance with the guidelines of the Committee for Research and Ethical Issues of IASP published in *Pain*, vol. 16, 1983, pp. 109–110. The animals were kept at 24±2 °C on a 12 h light/dark cycle (lights on at 07 h) and had food and water *ad libitum*.

Cell culture

NG108-15 cells, mouse neuroblastoma × rat glioma hybrid cells, were cultured in high glucose-containing Dulbecco's Modified Eagle's Medium (Wako Pure Chem.) supplemented with 0.1 mM hypoxanthine, 1 μM aminopterin, 16 μM thymidine, 50 $U \cdot mL^{-1}$ penicillin, 50 $\mu g \cdot mL^{-1}$ streptomycin and 10% FCS (Thermo Electron, Melbourne, Australia), as described previously (Nagasawa *et al.*, 2009). One day before

stimulation with db-cAMP or PGE_2, the concentration of FCS in the medium was decreased to 1%.

Dorsal root ganglion (DRG) neurons were obtained from male Wistar rats (400–500 g). The rats were killed by decapitation under anaesthesia with ether, and then, the DRGs at levels from L4 to L6 were quickly excised and incubated at 37°C for 60 min in Ham's F-12 medium (Wako Pure Chem.) containing 1% FCS and 2 mg·mL^{-1} collagenase (Wako Pure Chem.). The cells were washed and suspended in Ham's F-12 medium supplemented with 10% FCS and 50 U·mL^{-1} penicillin, 50 μg·mL^{-1} streptomycin. The cells were dispersed by pipetting several times at room temperature, seeded in plastic dishes (35 mm in a diameter) coated with poly-L-lysine and then cultured overnight in the 10% FCS-containing culture medium as mentioned above.

Whole-cell patch-clamp recordings

Whole-cell patch-clamp recordings in NG108-15 cells were performed as described previously (Kawabata *et al.*, 2007). NG108-15 cells (1×10^4 cells) were seeded in plastic dishes (35 mm in diameter) coated with poly-L-ornithine and cultured for a day in the above-mentioned culture medium containing 1% FCS. The culture medium was changed to an extracellular solution for patch-clamp experiments containing (mM): 97 *N*-methyl-D-glucamine, 10 $BaCl_2$, 10 HEPES, 40 tetraethylammonium chloride and 5.6 glucose (pH 7.4). After incubation for 30 min at 37°C, the cells were stimulated with db-cAMP or PGE_2 for 10 min at 37°C, as reported by Chemin *et al.* (2007). The reason why we used the extracellular solution for the incubation with db-cAMP or PGE_2 was (i) to avoid the effect of sudden change of the extracellular milieu by changing the culture medium to the extracellular solution for the patch-clamp measurements and (ii) to reduce the delay of the patch-clamp measurements after the incubation. FK506, a phosphatase inhibitor, at 1 μM was added just before stimulation with db-cAMP or PGE_2. db-cAMP, PGE_2 and FK506 were dissolved in distilled water, ethanol and DMSO, respectively, and added to the extracellular solution (1 mL) in volumes of 10, 1 and 1 μL respectively. Therefore, 'Vehicle' means addition of distilled water plus DMSO in db-cAMP (plus FK506) stimulation experiments and of ethanol plus DMSO in PGE_2 (plus FK506) stimulation experiments. After the 10 min stimulation, Ba^{2+} currents were recorded from randomly chosen cells at room temperature (22–25°C) using a whole-cell patch-clamp amplifier. A patch pipette was filled with an intracellular solution containing (mM): 140 CsCl, 4 $MgCl_2$, 5 EGTA and 10 HEPES (pH 7.2). The resistance of patch electrodes ranged from 3 to 7 MΩ. Series resistance was compensated by 80%, and current recordings were low-pass filtered (<5 kHz). The cell membrane voltage was held at –80 mV, and whole cell Ba^{2+} currents were elicited by step pulses of 200 ms duration from –120 to +40 mV with increments of 10 mV. Current density (pA/pF) was determined by dividing the currents by membrane capacitance. The T-channel-dependent currents (T currents) were determined as the difference between currents of the peak and 150 ms after the beginning of a step pulse at –20 mV (see Figure 1B, upper panel). The T currents for the steady-state inactivation curve were observed by stepping from various conditioning pre-pulses (–100 to –20 mV) of 1 s duration to a constant test pulse of –20 mV. Because the values of T currents in NG108-15 cells greatly varied with different passage numbers, effects of each stimulant and/or inhibitor were evaluated in the cells with the same passage number. Data were acquired and digitized with a Digidata interface (1322A, Axon Instruments, Foster City, CA) and analysed by a personal computer using pClamp8 software (Axon Instruments). The voltage dependencies of activation and steady-state inactivation were analysed by single Boltzmann distributions of the following forms: $G(V) = G_{max}/(1 + \exp[-(V - V_{1/2})/k])$ and $I(V) = I_{max}/(1 + \exp[(V - V_{1/2})/k])$, where G_{max} is the maximal conductance, I_{max} is the maximal activatable current, $V_{1/2}$ is the voltage in which half of the current is activated or inactivated and k represents the voltage dependence (slope) of the distribution. To determine the inhibitory effect of NNC 55-0396 (Figure 1B), after the control T currents were measured, NNC 55-0396 at 10 μM or vehicle was added to the extracellular solution, and T currents in the presence or absence (vehicle) of NNC 55-0396 were determined 10 min after the addition in the same cell. The T currents after addition of NNC 55-0396 or vehicle are shown as % of the control T currents in each cell.

Small DRG neurons (30 μm or less in a diameter) were selected, and T currents were measured, as described above, in the presence of nifedipine at 5 μM, ω-conotoxin GVIA at 1 μM and ω-conotoxin MVIIC at 1 μM, inhibitors of L-, N-, and P/Q-type Ca^{2+} channels respectively.

Immunoprecipitation and Western blotting

NG108-15 cells (2×10^6 cells) were seeded in plastic dishes (100 mm in diameter), grown for a day in the above mentioned culture medium containing 10% FCS and cultured in the 1% FCS-containing medium overnight. One hour after refreshing the 1% FCS-containing medium, the cells were stimulated with db-cAMP at 1 mM or a combination of PGE_2 at 10 μM and IBMX, a phosphodiesterase inhibitor, at 50 μM, and then incubated for 10 min at 37°C. It is to be noted that db-cAMP is capable of inhibiting phosphodiesterase, and that stimulation with the combination of PGE_2 and IBMX was more effective than PGE_2 alone in the preliminary experiments. FK506 was added 30 min before the stimulation to prevent dephosphorylation. Inhibitors of the downstream signals of PGE_2 or db-cAMP were also added 30 min before the stimulation. After the stimulation, the cells were harvested with the ice-cold lysis buffer [1% Nonidet® P-40 (Nacalai Tesque, Kyoto, Japan), 10 mM Tris–HCl, 150 mM NaCl, 0.5 mM EDTA, 10 mM NaF, pH 7.4] containing 1 mM Na_3VO_4 and 10% protease inhibitor cocktail (Sigma-Aldrich, cat# P8340). After centrifugation at 16 600 x *g* for 15 min at 4°C, the supernatant in a volume of 1 mL was incubated at 4°C with anti-$Ca_v3.2$ rabbit polyclonal antibody (Sigma-Genosis/Sigma-Aldrich) (10 μg of IgG protein) or anti-AKAP150 goat polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) (10 μg of IgG protein) for 1 h. The same concentrations of normal rabbit IgG (Cell Signaling Technology, Beverly, MA, USA) or normal goat IgG (Santa Cruz Biotechnology) were used as negative controls for the immunoprecipitation with anti-$Ca_v3.2$ and anti-AKAP150 antibodies respectively. After addition of 30 μL protein G-Sepharose (50%, v v^{-1}) (Sigma-Aldrich), the cells were further incubated for 1 h. The immunoprecipitates, collected by centrifugation, were washed five times with the lysis buffer, and then eluted from protein G-Sepharose by boiling

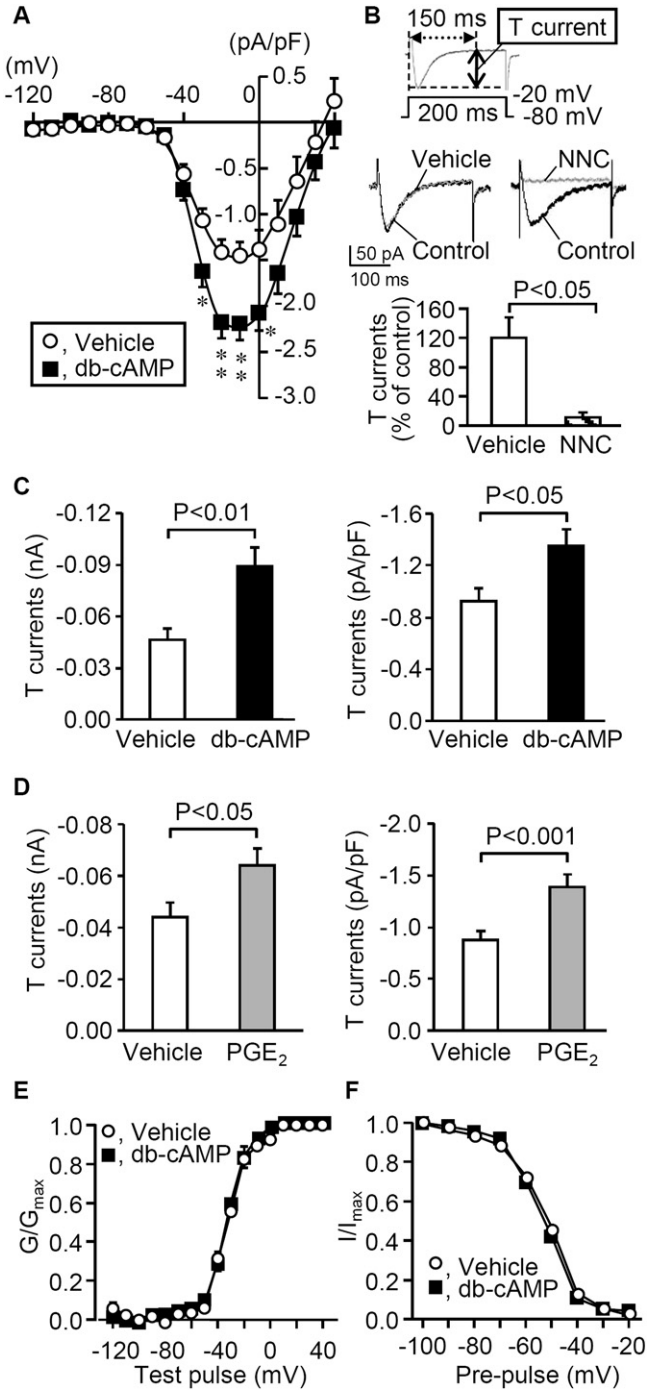

Figure 1

Increase in T currents caused by db-cAMP or PGE_2 in NG108-15 cells. (A) Averaged current–voltage relationships after stimulation with db-cAMP at 1 mM or vehicle at 37°C for 10 min in NG108-15 cells (leakage currents were subtracted). Step pulses from –120 to +40 mV were applied from a holding potential of –80 mV. (B) The T-channel blocker, NNC 55-0396 (NNC), at 10 μM abolished the T currents, which were measured as the difference between currents of the peak and 150 ms after the beginning of a test pulse at –20 mV. T currents in (B) are shown as % of the control currents before addition of NNC 55-0396 or vehicle. (C and D) Stimulation with db-cAMP at 1 mM (C) or PGE_2 at 10 μM (D) at 37°C for 10 min increased the T currents in the presence of FK506, a phosphatase inhibitor, at 1 μM. (E and F) db-cAMP did not alter the voltage-dependent activation (E) and steady-state inactivation (F) curves. Steady-state inactivation was determined by applying a pre-pulse of 1 s at various voltages immediately before the test pulse at –20 mV. The activation and steady-state inactivation curves were fitted according to the Boltzmann equation. $^*P < 0.05$, $^{**}P < 0.01$ versus vehicle. Data show the mean ± SEM for 21 (A), 4–6 (B), 23–24 (C), 28–31 (D) and 24 (E and F) different cells.

in the SDS sample buffer (2% SDS, 62.5 mM Tris–HCl, 10% glycerol, pH 6.8) for 5 min. Proteins in the supernatant were separated by SDS-PAGE and detected by Western blotting. The primary antibodies used in the present study were anti-$Ca_v3.2$ antibody (Sigma-Genosis/Sigma-Aldrich), anti-AKAP150 antibody (Millipore, Temecula, CA, USA) and, anti-phospho-(Ser/Thr) PKA substrate antibody (Cell Signaling Technology). Anti-rabbit and anti-goat HRP-linked IgG antibodies (Cell Signaling Technology) were used as secondary antibodies. Positive bands were developed by enhanced chemiluminescence detection (ECL, Western blotting detection reagent, Amersham Biosciences, Little-Chalfont, UK). The resulting films were scanned and quantified using densitometric software (Scion Image downloaded from http://scion-image.software.informer.com/).

Drug administration

The rat received i.pl. db-cAMP at 204 nmol per paw (100 μg per paw) or PGE_2 at 284 pmol per paw (100 ng per paw) in a volume of 100 μL. NNC 55-0396 at 10 nmol per paw, $ZnCl_2$ at 0.3 nmol per paw or verapamil at 10 nmol per paw was co-injected i.pl. with db-cAMP or PGE_2 in a volume of 100 μL. AKAPI at 0.1 nmol per paw or ω-conotoxin GVIA at 1 nmol per paw (3 μg per paw) in a volume of 10 μL were administered i.pl. 5–10 min before the i.pl. injection of db-cAMP or PGE_2. RQ-00015986-00 10 mg·kg^{-1} was administered p.o. 30 min before the i.pl. injection. Ethosuximide 50 mg·kg^{-1} or SB366791 500 μg·kg^{-1} were administered i.p. 5–10 min before the i.pl. injection of db-cAMP or PGE_2.

Pain behavioural assessment

Mechanical nociceptive threshold was determined by the paw pressure test, using an analgesia meter (MK-300, Muromachi Kikai Co., Tokyo, Japan) as described previously (Kawabata *et al.*, 2007). Pressure was applied to the hind paw of the rat at a linearly increasing rate of 30 g·s^{-1}, and the weight to induce an escaping activity was determined as nociceptive threshold. In case of the absence of response to a maximum pressure (500 g), the stimulation was stopped to prevent tissue damage. The data are presented as the percentage of the baseline threshold. In addition, the data are also presented as AUC (area under the curve) for the time course of the nociceptive threshold, since the AUC reflects the magnitude and persistence of the hyperalgesic or analgesic effects of drugs for a certain period of time, being beneficial to obtain reliable and reproducible data.

Determination of cutaneous blood flow of the hind paw in rats

Rats were anaesthetized with sodium pentobarbital (45 mg·kg^{-1}, i.p.) and cutaneous blood flow of the hind paw was measured by a laser Doppler flow meter (ALF-21; Advance Co., Tokyo, Japan). A probe (type C; Advance Co.) was placed on the plantar surface of the rat with double-faced adhesive. Verapamil 10 nmol per paw or vehicle (saline) in a volume of 10 µL was administered s.c. at a position about 5 mm far from the probe in the plantar. Cutaneous blood flow after the injection is expressed as % of the pre-injection values.

Statistics

All data are represented as mean ± SEM. The data were analysed statistically by Student's *t*-test for two-group comparisons and by Tukey's test for multiple comparisons. Significance was set at $P < 0.05$.

Results

AKAP-dependent enhancement of T currents through the PGE_2/EP_4 receptor/cAMP pathway in NG108-15 cells and rat DRG neurons

As reported previously (Chemin *et al.*, 2002; Kawabata *et al.*, 2007; Nagasawa *et al.*, 2009), the undifferentiated NG108-15 cells showed typical T currents, characterized by their activation in response to low-voltage pulses (the threshold of –70 mV; the maximal currents at –20 to –10 mV) (Figure 1A) and transient currents (Figure 1B, the top panel), but not high-voltage-activated currents. To confirm that the currents are actually T-channel-dependent, the effect of NNC 55-0396, a selective T-channel inhibitor, was determined. The T currents (% of the pre-drug control) greatly decreased after addition of NNC 55-0396 10 µM, but not vehicle, showing that the T currents were abolished by NNC 55-0396 (Figure 1B, bottom panel).

db-cAMP enhanced the currents in response to pulses at –30 to 0 mV (Figure 1A) but had little effect on the voltage dependence of activation (Figure 1E, $V_{1/2}$: vehicle, –30.21 ± 1.64 mV; db-cAMP, –30.70 ± 1.38 mV. k: vehicle, 8.52 ± 0.75 mV; db-cAMP, 9.38 ± 0.68 mV) and steady-state inactivation (Figure 1F, $V_{1/2}$: vehicle, –54.44 ± 1.04 mV; db-cAMP, –54.13 ± 0.85 mV. k: vehicle, 7.96 ± 0.66 mV; db-cAMP, 7.16 ± 0.53 mV). The T currents were significantly enhanced by the stimulation with db-cAMP or PGE_2 (Figure 1C, D), The facilitation of T currents by db-cAMP and PGE_2 was blocked by AKAP St-Ht31 inhibitor peptide (AKAPI) at 25 µM that dissociates PKA from AKAPs (Vijayaraghavan *et al.*, 1997) (Figure 2A, C) and KT5720, an inhibitor of PKA, at 10 µM (Figure 2B, D). The facilitating effect of PGE_2 on T currents was also blocked by RQ-00015986-00, an antagonist of EP_4 receptors, at 10 µM (Figure 2E).

We next examined whether the PGE_2/cAMP pathway also modulates T channels in rat isolated DRG neurons. In small DRG neurons (30 µm or less in diameter), voltage-dependent currents with a threshold at –60 mV and peak at –10 mV were detected in the presence of nifedipine 5 µM, ω-conotoxin GVIA 1 µM and ω-conotoxin MVIIC 1 µM, to remove contamination of HVA currents (Figure 3A). Stimulation with db-cAMP 1 mM for 10 min at 37°C markedly enhanced the currents (Figure 3A). T currents, calculated as shown in Figure 1B, were significantly increased by the stimulation with db-cAMP, an effect suppressed by AKAPI (Figure 3B).

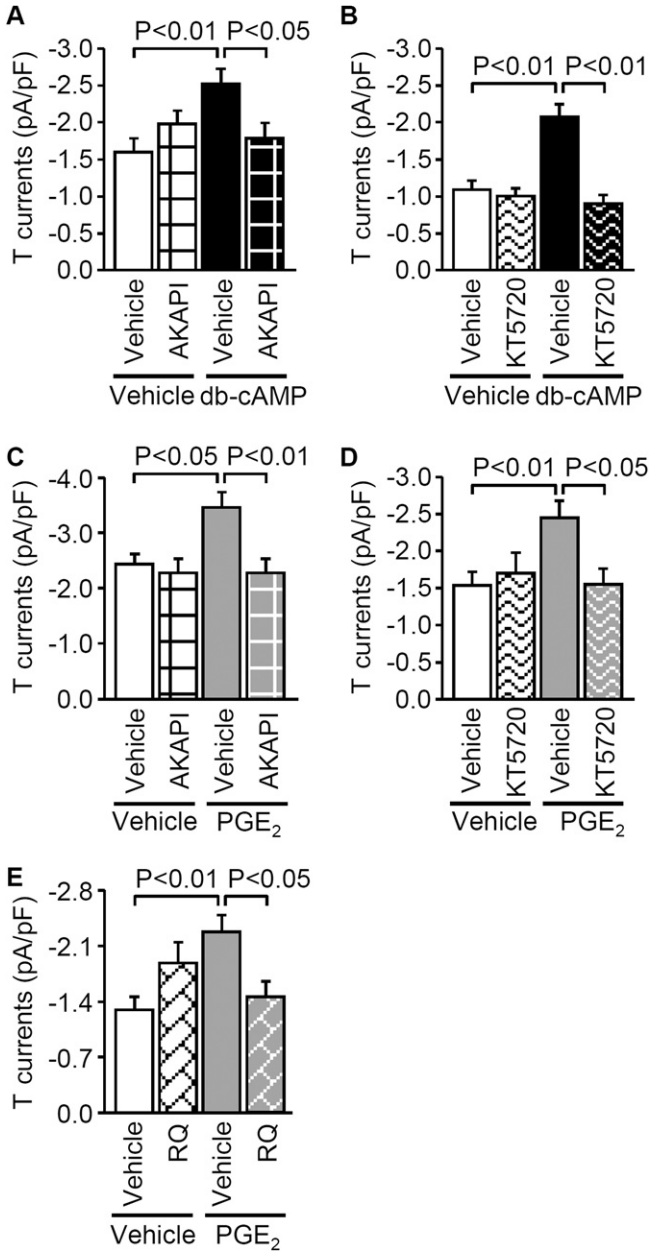

Figure 2

Effects of AKAPI, an inhibitor of PKA anchoring to AKAP, KT5720, a PKA inhibitor, or RQ-00015986-00, an EP_4 receptor antagonist, on the increase in T currents caused by db-cAMP or PGE_2 in NG108-15 cells. AKAPI 25 µM (A, C), KT5720 10 µM (B, D) or RQ-00015986-00 (RQ) 10 µM (E) was added 30 min before 10 min stimulation with db-cAMP 1 mM (A, B) or PGE_2 10 µM (C, D, E) in the presence of FK506 1 µM. Data show the mean ± SEM for 35–41 (A), 21 (B), 29–32 (C), 22–26 (D) and 20–21 (E) different cells.

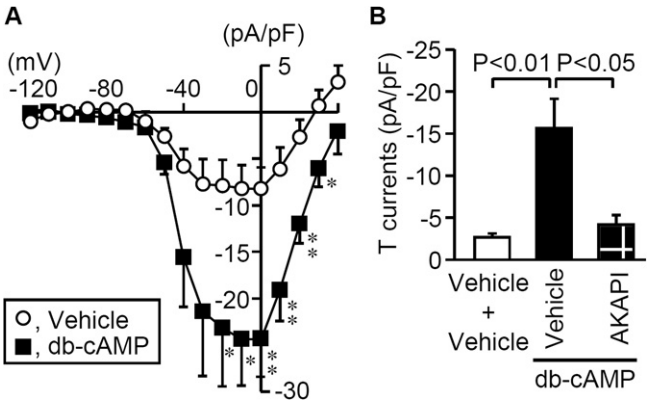

Figure 3

Increase in T currents caused by db-cAMP and its inhibition by AKAPI in rat small DRG neurons. (A) Averaged current–voltage relationships after stimulation with db-cAMP at 1 mM or vehicle at 37°C for 10 min in the presence of FK506 1 μM, a phosphatase inhibitor (leakage currents were subtracted). Test pulses of 200 ms duration from −120 to +40 mV were applied from a holding potential of −80 mV, in the presence of HVA Ca^{2+} channel inhibitors, as described in the Methods section. (B) AKAPI 25 μM was added 30 min before stimulation with db-cAMP. T currents were calculated as described in Figure 1B. $*P < 0.05$, $**P < 0.01$ versus vehicle. Data show the mean ± SEM for 10–11 (A) and 9–12 (B) different neurons.

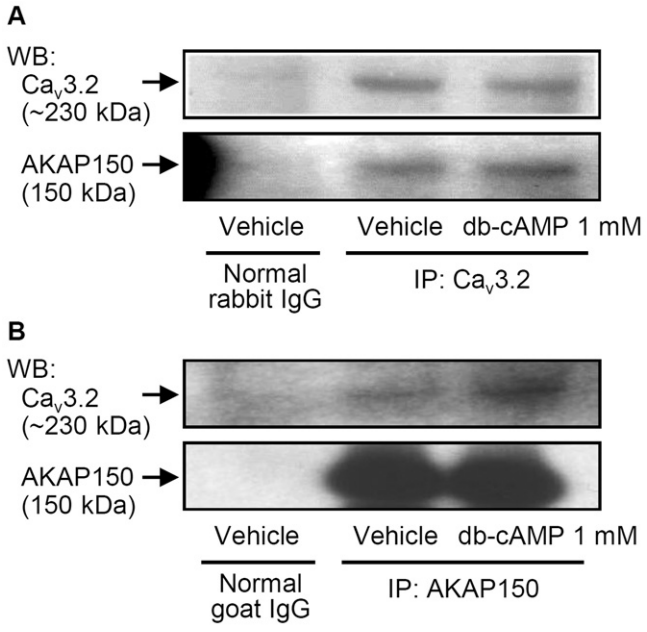

Figure 4

Co-immunoprecipitation of $Ca_v3.2$ T channels and AKAP150 in NG108-15 cells. NG108-15 cells were stimulated with db-cAMP 1 mM in the presence of FK506 1 μM for 10 min at 37°C and subjected to immunoprecipitation (IP) with anti-$Ca_v3.2$ antibody or normal rabbit IgG (A) and anti-AKAP150 antibody or normal goat IgG (B).

Molecular association of $Ca_v3.2$ with AKAP150 and its phosphorylation by db-cAMP or PGE_2 in NG108-15 cells

To determine the possible direct molecular association between $Ca_v3.2$ and AKAP150, immunoprecipitation with the anti-$Ca_v3.2$ or anti-AKAP150 antibodies was performed in NG108-15 cells. AKAP150 was actually co-immunoprecipitated with $Ca_v3.2$, regardless of stimulation with db-cAMP at 1 mM for 10 min at 37°C (Figure 4A). The protein complex of $Ca_v3.2$ and AKAP150 was also observed in the immunoprecipitates with the anti-AKAP150 antibody (Figure 4B). Stimulation with db-cAMP significantly increased phosphorylation of Ser/Thr residues in $Ca_v3.2$ protein (~230 kDa), an effect reversed by AKAPI (Figure 5A). Stimulation with PGE_2 in combination with IBMX also significantly increased phosphorylation levels of $Ca_v3.2$, an effect inhibited by RQ-00015986-00 (Figure 5B).

Involvement of $Ca_v3.2$ T channels in the mechanical hyperalgesia caused by i.pl. administration of db-cAMP or PGE_2 in rats

Administration of db-cAMP 204 nmol per paw i.pl. (100 μg per paw) or PGE_2 284 pmol per paw (100 ng per paw) caused mechanical hyperalgesia in rat hind paw (Figure 6A, B). The db-cAMP- and PGE_2-induced mechanical hyperalgesia reached the peak at 1.5 and 2 h, and lasting until 2.5 and 4 h after administration respectively. When the AUC was calculated from the time-nociceptive threshold curves between 1.5 and 2.5 h and between 2 and 4 h after i.pl. db-cAMP and PGE_2, respectively, the AUC values were significantly lower than those of each control group (Figure 6C, D, E). AKAPI, an inhibitor of AKAP acting as a scaffold for PKA, at 0.1 nmol per paw, when administered i.pl. 5 min before db-cAMP or PGE_2, prevented the development of hyperalgesia (Figure 6C, E). The PGE_2-induced mechanical hyperalgesia was also significantly inhibited by administration of RQ-00015986-00, the EP_4 receptor antagonist, at 10 $mg \cdot kg^{-1}$ p.o. (Figure 6D).

NNC 55-0396, 10 nmol per paw, the T-channel blocker, co-administered i.pl. with db-cAMP, completely inhibited the db-cAMP-induced hyperalgesia (Figure 7A). Another T-channel blocker, ethosuximide, at 50 $mg \cdot kg^{-1}$ when administered i.p., also exhibited an inhibitory effect (Figure 7B). Administration of $ZnCl_2$ i.pl. at 0.3 nmol per paw, which is known to selectively block $Ca_v3.2$ among the three isoforms of T channels (Nelson *et al.*, 2007), also inhibited the mechanical hyperalgesia caused by i.pl. db-cAMP (Figure 7C). It was noteworthy that the db-cAMP-induced hyperalgesia was also inhibited by i.pl. ω-conotoxin GVIA, an N-type Ca^{2+} channel (N-channel) inhibitor, at 1 nmol per paw (Figure 7D). Neither NNC 55-0396, ethosuximide, $ZnCl_2$ nor ω-conotoxin GVIA, induced hypoalgesia by themselves (Figure 7A–D). In contrast, i.pl. administration of verapamil, an L-type Ca^{2+} channel blocker, at 10 nmol per paw had no effect on the hyperalgesia caused by i.pl. db-cAMP (Figure 7E), although it significantly increased cutaneous blood flow in the plantar region (Figure 7F). The mechanical hyperalgesia caused by i.pl. PGE_2 was also abolished by i.pl. administration of NNC 55-0396 or $ZnCl_2$ (Figure 8A, B).

Finally, i.p. administration of SB366791, an antagonist of TRPV1 channels, at 500 $\mu g \cdot kg^{-1}$ failed to inhibit the mechanical hyperalgesia caused by i.pl. db-cAMP or PGE_2 (Figure 9).

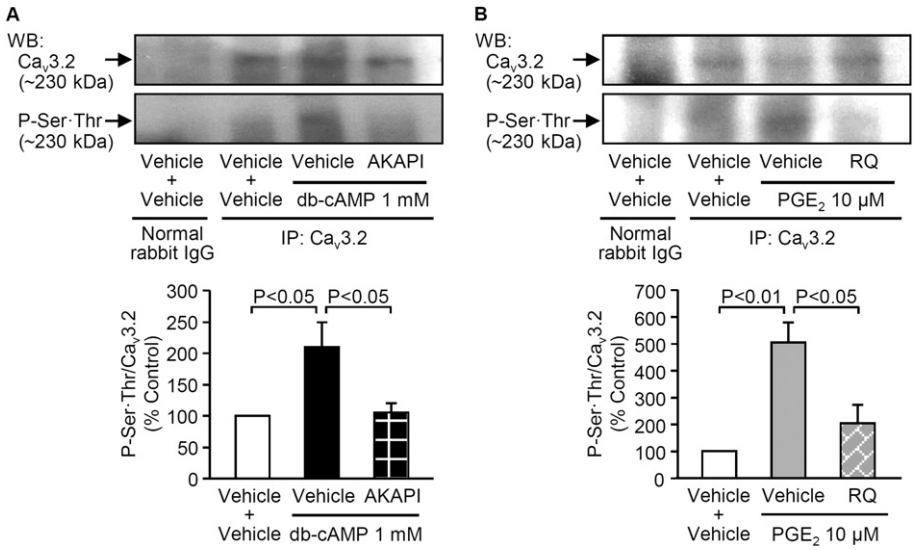

Figure 5

Phosphorylation of $Ca_v3.2$ caused by db-cAMP or PGE_2 in NG108-15 cells. NG108-15 cells were stimulated with db-cAMP 1 mM (A) or PGE_2 10 μM in combination with IBMX 50 μM (B) in the presence of FK506 1 μM for 10 min at 37°C and subjected to immunoprecipitation with anti-$Ca_v3.2$ antibody or normal rabbit IgG. AKAPI 25 μM (A) and RQ-00015986-00 10 μM, an EP_4 receptor antagonist, (B) were added 30 min before the stimulation. Phosphorylated levels of Ser/Thr (P-Ser/Thr)/$Ca_v3.2$ are expressed as % of the values of control (vehicle + vehicle). Data show the mean ± SEM from four experiments.

Discussion and conclusions

Our data obtained from the experiments using NG108-15 cells and rat isolated DRG neurons demonstrate the direct molecular association between $Ca_v3.2$ T channels and AKAP150, and reveal that $Ca_v3.2$ T channels are phosphorylated and sensitized by the PGE_2/EP_4 receptor/cAMP pathway in an AKAP-dependent manner. Furthermore, the results from the *in vivo* experiments in rats suggest that T channels, most probably of the $Ca_v3.2$ isoform, AKAP and EP_4 receptors are involved in the development of mechanical hyperalgesia caused by db-cAMP and/or by PGE_2 in rat hind paw. Together, we propose that AKAP-dependent phosphorylation and sensitization of $Ca_v3.2$ through the EP_4 receptor/cAMP/PKA pathway play a role in the PGE_2-induced mechanical hyperalgesia in rat hind paw.

The NG108-15 cells employed in the present study, known as neuron-like model cells, are particularly useful for the study of $Ca_v3.2$ T channels, because undifferentiated NG108-15 cells abundantly express T channels, especially of the $Ca_v3.2$ isoform, but not HVA Ca^{2+} channels (Chemin *et al.*, 2002; Nagasawa *et al.*, 2009). In addition, NG108-15 cells produce cAMP in response to PGE_2 (Gylys *et al.*, 1997). Thus, NG108-15 cells are valuable for studying the functional linkage among $Ca_v3.2$, cAMP and PGE_2. Our electrophysiological and immunoprecipitation/immunoblotting studies employing undifferentiated NG108-15 cells clearly demonstrate that activation of PKA with db-cAMP causes phosphorylation and sensitization of $Ca_v3.2$ T channels, which is consistent with findings from previous studies using $Ca_v3.2$-transfected cells (Kim *et al.*, 2006; Chemin *et al.*, 2007; Hu *et al.*, 2009). Furthermore, our data show that PGE_2 mimics the facilitating effect of db-cAMP on T currents and phosphorylation of $Ca_v3.2$ via activation of EP_4 receptors that are coupled to Gs protein. Most interestingly, our study, for the first time to our knowledge, provides evidence that AKAP, a scaffolding protein for PKA, is essential for sensitization of T channels by db-cAMP or PGE_2, using AKAP St-Ht31 inhibitor peptide (N-stearate-DLIEEAASRIVDAVIEQVKAAGAY), which encompasses the PKA regulatory subunit binding site of AKAPs, and is widely used to competitively dissociate PKA/AKAP complexes in the range 5–50 μM (Vijayaraghavan *et al.*, 1997; Welch *et al.*, 2010). In addition, we also showed that AKAP150, the rodent orthologue of human AKAP79, forms a molecular complex with $Ca_v3.2$ T channels, regardless of stimulation with db-cAMP. Such AKAP79/150-dependent modulation of ion channels by PKA or PKC is well documented for TRPV1 channels that play a crucial role in PGE_2-induced thermal hyperalgesia (Jeske *et al.*, 2008; 2009; Schnizler *et al.*, 2008; Zhang *et al.*, 2008). $Ca_v3.2$ T channels also appear to be phosphorylated by PKC, whereas there is conflicting evidence that PKC sensitizes (Park *et al.*, 2006; Chemin *et al.*, 2007) and desensitizes (Rangel *et al.*, 2010; Zhang *et al.*, 2011) T channels in different experimental conditions. The physiological importance of our findings in the experiments using NG108-15 cells is strongly supported by the AKAP-dependent facilitation of T currents by db-cAMP in rat isolated DRG neurons in the present study.

Our *in vivo* inhibition experiments using two distinct inhibitors of T channels, NNC 55-0396 and ethosuximide, provide novel evidence for the involvement of T channels in the mechanical hyperalgesia caused by i.pl. administration of PGE_2 or db-cAMP in rats. The T channels involved in the PGE_2-induced mechanical hyperalgesia are probably of the $Ca_v3.2$ isoform as $ZnCl_2$, known to inhibit $Ca_v3.2$, but not $Ca_v3.1$ or $Ca_v3.3$ had an inhibitory effect (Nelson *et al.*, 2007).

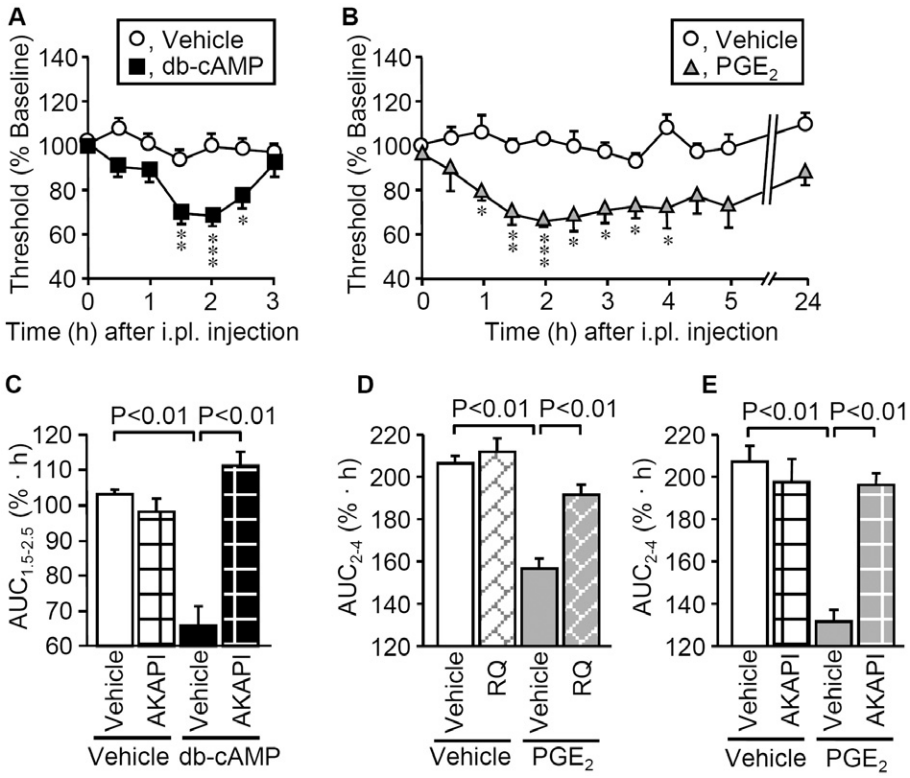

Figure 6

Mechanical hyperalgesia induced by i.pl. administration of db-cAMP or PGE_2 in rats, and its inhibition by AKAPI or RQ-00015986-00, an EP_4 receptor antagonist. (A, B) Time course of mechanical nociceptive threshold after i.pl. db-cAMP, 204 nmol per paw (100 μg per paw), or PGE_2, 284 pmol per paw (100 ng per paw). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle. (C, D, E) Inhibitory effects of i.pl. administration of AKAPI or oral administration of RQ-00015986-00 (RQ). The AUC was calculated from the time–threshold curves between 1.5 and 2.5 h after i.pl. db-cAMP (C) or between 2 and 4 h after i.pl. PGE_2 (D, E). AKAPI, 0.1 nmol per paw (0.3 μg per paw), was administered i.pl. 5 min before i.pl. db-cAMP or PGE_2 (C, E). RQ-00015986-00 10 mg·kg^{-1} was administered orally 30 min before injection of PGE_2 (D). Data show the mean ± SEM for 4 (A, C, E), 4–6 (B) or 4–7 (D) rats.

It has been reported that the IC_{50} values for Zn^{2+} inhibition of $Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$ are 81.7, 0.78 and 158.6 μM respectively (Traboulsie *et al.*, 2007). The final concentration of $ZnCl_2$ used for i.pl. administration in the present study was 3 μM and this would selectively inhibit $Ca_v3.2$, but not $Ca_v3.1$ or $Ca_v3.3$. Our findings that the T-channel inhibitors, NNC 55-0396 and ethosuximide, did not affect the basal pain but suppressed the hyperalgesia induced by db-cAMP or PGE_2, do not accord with those from two previous studies, which showed that knockout of $Ca_v3.2$ (Choi *et al.*, 2007) or knockdown of $Ca_v3.2$ (Bourinet *et al.*, 2005) induced a basal mechanical analgesia. There are many independent studies including our previous works, in which the effects of systemic or i.pl. administration of T-channel blockers were examined in normal and hyperalgesic animals, and most of them have shown that T-channel blockers prevent hyperalgesia or allodynia, but do not affect the basal pain (Dogrul *et al.*, 2003; Todorovic *et al.*, 2004; Choe *et al.*, 2011). It is likely that T-channel blockers might preferentially target the peripheral endings of the nociceptors, while genetic knockout or knockdown of $Ca_v3.2$ deletes or silences the expression of $Ca_v3.2$ in both peripheral and central endings of the nociceptors, and also in spinal or supraspinal neurons. These differences might be the reason why knockout or knockdown of $Ca_v3.2$, but not T-channel blockers, reduces basal pain. Nonetheless, it is noteworthy that many studies including our previous works have reported that knockout or knockdown of $Ca_v3.2$ did not affect the basal pain, but prevented hyperalgesia or allodynia (Kawabata *et al.*, 2007; Latham *et al.*, 2009; Lee *et al.*, 2009; Maeda *et al.*, 2009; Chen *et al.*, 2010; Takahashi *et al.*, 2010; Okubo *et al.*, 2011; 2012), and the reason for this discrepancy is still unresolved.

As ω-conotoxin GVIA, an N-channel blocker, had an inhibitory effect in the present study, N channels may also contribute to the cAMP-mediated mechanical hyperalgesia (see Figure 7D). Some studies have shown that activation of the cAMP/PKA pathway facilitates the function of N channels (Kohno *et al.*, 2003; Rola *et al.*, 2008). On the other hand, in a *Xenopus* oocyte expression system, the PKA-induced potentiation of N-channel currents appears to be much smaller than that of Q-channel currents (Kaneko *et al.*, 1998). To our knowledge, there is no report suggesting direct AKAP-dependent modulation of N channels by PKA. The possibility that N channels are activated secondarily by the PKA-dependent activation of T channels and this contributes to the development of hyperalgesia cannot be ruled out. On the

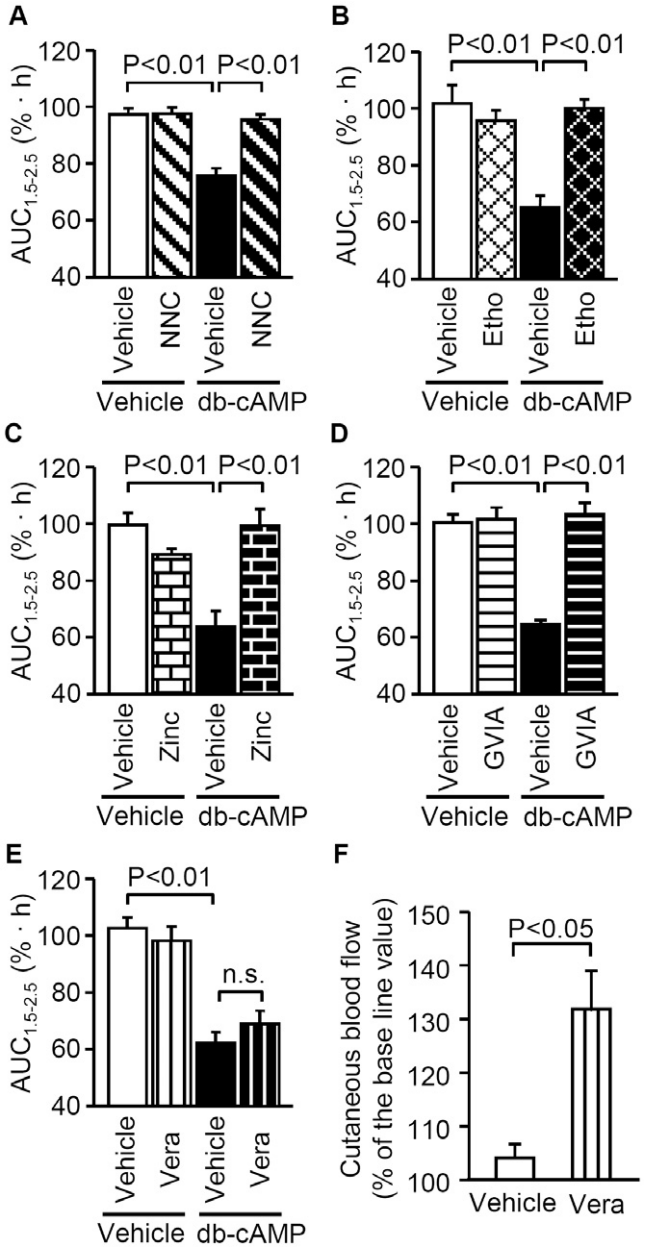

Figure 7

Effects of NNC 55-0396 and ethosuximide, T-channel blockers, zinc chloride, known to inhibit $Ca_v3.2$, but not $Ca_v3.1$ or $Ca_v3.3$, ω-conotoxin GVIA, an N-channel blocker, and verapamil, an L-type Ca^{2+} channel blocker, on the db-cAMP-induced mechanical hyperalgesia in rats. The AUC was calculated from the time–threshold curves between 1.5 and 2.5 h after i.pl. db-cAMP. NNC 55-0396 (NNC) at 10 nmol per paw (A), zinc chloride (Zinc) at 0.3 nmol per paw (C), or verapamil (Vera) at 10 nmol per paw (E) was co-injected i.pl. with db-cAMP at 204 nmol per paw. Ethosuximide (Etho) 50 $mg \cdot kg^{-1}$ was administered i.p. 10 min before i.pl. db-cAMP (B). ω-Conotoxin GVIA at 1 nmol per paw (3 μg per paw) was administered i.pl. 10 min before the stimulation with db-cAMP (D). (F) The increase in cutaneous blood flow caused by i.pl. verapamil, 10 nmol per paw, in the hind paw of rats. Data show the mean ± SEM for 5 (A), 4 (B, D) or 6 (C, E, F) rats.

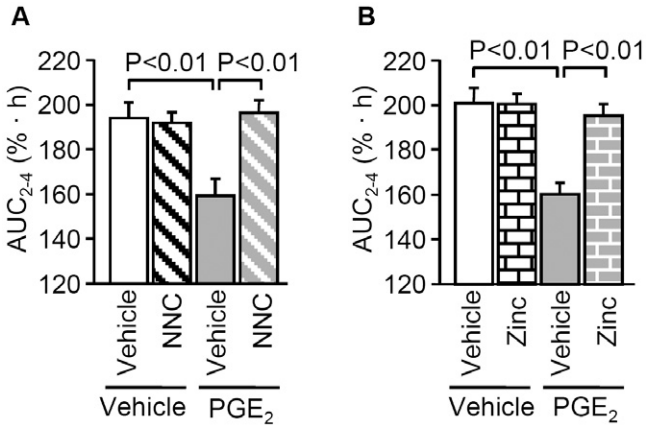

Figure 8

Effects of NNC 55-0396 or zinc chloride on the PGE_2-induced mechanical hyperalgesia in rats. The AUC was calculated from the time-threshold curves between 2 and 4 h after i.pl. PGE_2. NNC 55-0396 (NNC) at 10 nmol per paw (A) or zinc chloride (Zinc) at 0.3 nmol per paw (B) was co-injected i.pl. with PGE_2, 284 pmol per paw. Data show the mean ± SEM for 10 (A) or 4 (B) rats.

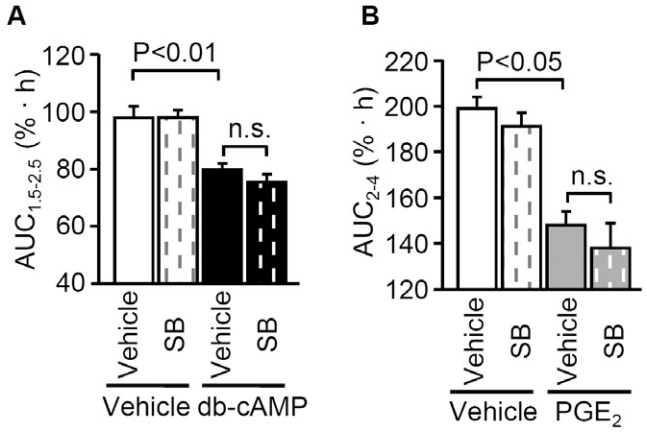

Figure 9

Lack of effect of a TRPV1 receptor antagonist, SB366791 (SB), on the mechanical hyperalgesia caused by db-cAMP or PGE_2 in rats. The AUC was calculated from the time–threshold curves between 1.5 and 2.5 h and between 2 and 4 h after i.pl. db-cAMP (A) and PGE_2 (B) respectively. SB366791 (SB) 500 $\mu g \cdot kg^{-1}$ was administered i.p. 5 min before i.pl. injection of db-cAMP at 204 nmol per paw or PGE_2 at 284 pmol per paw. Data show the mean ± SEM for four rats. n.s., not significant.

other hand, it is clear that L-type Ca^{2+} channels are not involved in the mechanical hyperalgesia mediated by the PGE_2/cAMP pathway, because verapamil exhibited no inhibitory effects on the hyperalgesia at the dose that increased the cutaneous blood flow (see Figure 7E, F). In addition, the involvement of TRPV1 channels in the PGE_2-induced mechanical hyperalgesia can also be ruled out by our data, which showed that the hyperalgesia was not suppressed by SB366791, a TRPV1 inhibitor, at 500 $\mu g \cdot kg^{-1}$, a dose that completely inhibits the increase in blood flow induced by

capsaicin, a TRPV1 agonist (Varga *et al.*, 2005). TRPA1 is also involved in thermal hyperalgesia. However, to our knowledge, there is no evidence that TRPA1 contributes to the PGE_2-induced thermal or mechanical hyperalgesia, although this possibility remains to be tested by future in-depth studies.

Among the four subtypes of PGE_2 receptors, EP_2 and EP_4 receptors are coupled to the Gs protein and, upon activation, stimulate the adenylyl cyclase/cAMP/PKA pathway (Narumiya and FitzGerald, 2001; Kawabata, 2011). Given the inhibitory effects of a highly selective EP_4 receptor antagonist, RQ-00015986-00 (CJ-042794), that is at least 200-fold more selective for EP_4 than EP_1, EP_2 or EP_3 (Murase *et al.*, 2008b), EP_4 receptors are considered to mediate both PGE_2-induced facilitation of T currents in NG108-15 cells and mechanical hyperalgesia in rat hind paw. There is plenty of evidence that EP_4 receptors play a pivotal role in peripheral inflammatory pain (Nakao *et al.*, 2007; Clark *et al.*, 2008; Murase *et al.*, 2008a; Colucci *et al.*, 2010). Thus, RQ-00015986-00 is considered useful as an orally available analgesic with minimal side effects, compared with non-steroidal anti-inflammatory drugs that inhibit production of all prostanoids. The present evidence that T channels are downstream of EP_4 receptors suggests that selective T-channel blockers might also be useful for treatment of inflammatory pain. It is also likely that activation of EP_2 receptors induces PKA-dependent sensitization of T channels, whereas EP_2 receptors appear to play a role in the processing of inflammatory pain signals in the spinal cord, but not in the peripheral tissues (Reinold *et al.*, 2005; Kawabata, 2011). Activation of EP_1 receptors, coupled to Gq protein, causes PKC-dependent sensitization of TRPV1 channels, leading to thermal hyperalgesia (Moriyama *et al.*, 2005; Zhang *et al.*, 2008; Jeske *et al.*, 2009). As mentioned above, PKC may also facilitate the functions of $Ca_v3.2$ T channels, although conflicting evidence has also been reported (Park *et al.*, 2006; Chemin *et al.*, 2007; Rangel *et al.*, 2010; Zhang *et al.*, 2011). Actually, there is evidence that PKC contributes to the PGE_2-induced mechanical hyperalgesia in rats (Sachs *et al.*, 2009). However, it is still open to question whether the PGE_2/EP_1 receptor/PKC pathway-mediated hyperalgesia involves the sensitization of $Ca_v3.2$. Our study is now in progress to clarify the involvement of PKC modulation of $Ca_v3.2$ functions in processing of pain signals.

In addition to PKA, Epac (exchange proteins activated by cAMP) functions as another downstream pathway of cAMP (Holz *et al.*, 2006). Interestingly, it has been reported that an Epac agonist, 8CPT-2′-*O*-methyl-cAMP, increased mRNA and Ca^{2+} currents of $Ca_v3.2$ in bovine adrenal zona fasciculate cells (Liu *et al.*, 2010). However, the effect of the Epac agonist on $Ca_v3.2$ was observed 72 h, but not 48 h, after the stimulation in those cells. In contrast, our study showed that the increase in T currents and phosphorylation of $Ca_v3.2$ occurred just after 10 min stimulation with db-cAMP or PGE_2. Considering the rapid onset of the effects and their prevention by AKAPI and KT5720 that inhibit PKA through different mechanisms, PKA, but not Epac, appears to play a major role in the increased T-current induced by db-cAMP or PGE_2.

In conclusion, $Ca_v3.2$ T channels are considered to be phosphorylated and sensitized by the PGE_2/EP_4 receptors/cAMP/PKA pathway in an AKAP-dependent manner, contributing to the PGE_2-induced mechanical hyperalgesia. Our study thus strongly suggests that selective T-channel blockers as well as selective EP_4 receptor antagonists could be therapeutically useful as analgesics for the treatment of inflammatory pain.

Acknowledgements

This research was supported in part by Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science and by 'Antiaging Center Project' for Private Universities from Ministry of Education, Culture, Sports, Science and Technology, 2008–2012.

Conflicts of interest

There are no conflicts of interest.

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