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RESEARCH ARTICLE

Desensitization of cold- and menthol-sensitive rat dorsal root ganglion neurones by inflammatory mediators

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Abstract The interaction between cold sensitivity and inflammation in mammals is not entirely understood. We have used adult rat dorsal root ganglion neurones in primary culture together with calcium microfluorimetry to assess the effects of selected inflammatory mediators on cold responses of cold- and menthol-sensitive (most likely TRPM8-expressing) neurones. We observed a high degree of functional co-expression of TRPM8, the receptors for the inflammatory agents bradykinin, prostaglandin E_2 and histamine, and TRPA1 in cultured sensory neurones. Treatment with either bradykinin or prostaglandin E_2 led to a reduction in the amplitude of the response to cooling and shifted the threshold temperature to colder values, and we provide evidence for a role of protein kinases C and A, respectively, in mediating these effects. In both cases the effects were mainly restricted to the subgroups of cold- and menthol-sensitive cells which had responded to the application of the inflammatory agents at basal temperature. This desensitization of cold-sensitive neurones may enhance inflammatory pain by removing the analgesic effects of gentle cooling.

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Abbreviations

DRG	Dorsal root ganglia
CMS	Cold- and menthol-sensitive neurones
BK	Bradykinin
PGE_2	Prostaglandin E_2
HA	Histamine
CA	Cinnamon aldehyde
PKA	Protein kinase A
PKC	Protein kinase C
8-Br-cAMP	8 Bromo cyclic AMP

Introduction

The molecular events implicated in sensory transduction in mammalian specialised nerve endings as well as the alterations induced in pathological states have been the focus of intensive research in the past decades. Inflammation is a widespread condition accompanying tissue damage, in which the release of a variety of chemical agents (bradykinin, prostaglandins, histamine, nerve growth factor, ATP, serotonin, protons, etc...) leads to sensitisation of primary nociceptive neurones, generating abnormal responses to mild stimuli (allodynia) and exaggerated pain perception (hyperalgesia). Increased sensitivity to thermal (both heat and cold) stimuli is known to be associated with inflammatory states and a clearer picture of the mechanisms underlying these changes has recently emerged, particularly for heat responses. A major player in hypersensitivity to heat appears to be TRPV1 (the capsaicin receptor, Caterina et al. 1997), as mice lacking the

receptor do not show any sign of inflammatory heat hyperalgesia (Davis et al. 2000; Caterina et al. 2000). The signalling pathways implicated in the enhanced TRPV1 thermal sensitivity with inflammation have been extensively studied and important results have been reported, although complete consensus has not been reached. However, both bradykinin and prostaglandin E_2 have been shown to sensitise TRPV1-mediated capsaicin and heat responses (Cesare and McNaughton 1996; Lopshire and Nicol 1998), and the signalling pathways involved have also been described to a great extent (Vellani et al. 2001; Chuang et al. 2001; Bhave et al. 2002). It was recently shown that histamine activates phospholipase A_2 and sensitises TRPV1 via the lipoxygenase pathway (Kim et al. 2004).

Such a plethora of data on alterations induced by inflammatory agents on the biophysical properties of cold-activated ion channels is still missing. Two ion channels activated by cooling have been cloned recently: TRPM8, the cold and menthol receptor (McKemy et al. 2002; Peier et al. 2002), and TRPA1 (Story et al. 2003), which requires stronger cooling for activation and is also sensitive to pungent compounds such as cinnamon aldehyde, allyl isothiocyanate (mustard oil) and allicin (the active compound in garlic). However, the activation of TRPA1 by cooling, as well as its proposed role in mediating cold nociception are still controversial issues (Jordt et al. 2004; Babes et al. 2004; Nagata et al. 2005; Bautista et al. 2006). Interestingly, TRPA1 is also activated by BK via the PLC pathway and its expression in DRG neurones is increased in inflammation and nerve injury, raising the hypothesis of an involvement in inflammatory cold pain (Bandell et al. 2004; Obata et al. 2005). Very recent work has focused on TRPM8 and its modulation. Two studies described a positive modulation of the channel by the membrane phsopholipid PIP_2. Activation of the PLC pathway, followed by PIP_2 cleavage and depletion from the membrane leads to an inhibition of TRPM8 (Liu and Qin 2005; Rohacs et al. 2005). Two other groups reported that stimulation of PKC also inhibits TRPM8 (Premkumar et al. 2005; Abe et al. 2005). Importantly, Premkumar et al. (2005) described the inhibitory effect of PKC on the current activated by either cold or menthol in TRPM8-expressing *Xenopus* oocytes, and also demonstrated that BK inhibits menthol-induced currents and calcium transients in cultured DRG neurones via PKC-mediated dephosphorylation of native TRPM8.

The aim of our work was to investigate the putative modulatory effects of selected inflammatory mediators on the activation of native DRG neurones by cooling. Our results confirm and extend previous work, providing evidence towards a high degree of expression of BK and PGE_2 receptors in cold- and menthol-sensitive (CMS) neurones in which they mediate an inhibitory effect.

Materials and methods

Dorsal root ganglion (DRG) neurones were obtained from spinal levels L1-S1 from adult Wistar rats (150–170 g) as described elsewhere (Reid et al. 2002). Briefly, the animals were killed by CO_2 inhalation followed by decapitation, following a UK Home Office approved (Schedule 1) procedure. DRGs were removed and incubated in a mixture of 1 mg/ml Collagenase (type IX, Sigma) and 3 mg/ml Dispase (nonspecific protease, Sigma) in IncMix solution (see Solutions, below) for 1 h at 37°C. After trituration the dissociated cells were plated onto 25 mm borosilicate glass coverslips (0.17 mm thick) which had been treated with poly-D-lysine (0.1 mg/ml for 30 min), and cultured (37°C, 5% CO_2 in air) in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium with 10% horse serum and 50 μg/ml gentamicin. NGF was not added to the culture medium. The neurones were used for recording the day after the dissection (between ~12 and 24 h after plating). All chemicals used for cell culture were from Sigma.

Before recording, coverslips with attached neurones were incubated for 30 min at 37°C in standard extracellular solution (see below) containing 2 μM Calcium Green-1 AM and 0.02% Pluronic F-127 (both from Molecular Probes, Leiden, the Netherlands). The cells were left to recover for another 30 min before use. Coverslips were then mounted in a Teflon chamber (MSC TD, Digitimer, Welwyn Garden City, UK) on the stage of an Olympus IX70 inverted microscope and adapted for 5 min at a base temperature of ~32°C, before applying cooling stimuli. Temperature was controlled by local superfusion with a Peltier-based system described elsewhere (Reid et al. 2001). In order to measure the temperature experienced by the neurones, after the experiment a miniature thermocouple (1T-1E, Physitemp, Clifton, NJ, USA) was placed where the cells had been and the thermal stimuli were repeated. The reproducibility of the thermal stimuli is very good. The thermal stimulus consisted of a 40 s cooling ramp from a base temperature of ~32 to ~18°C. Fluorescence changes were recorded with a CCD camera (Cohu 4910, Pieper GmbH, Schwerte, Germany). The cells were illuminated with a 100-W halogen lamp and filter wheel (Cairn Research, Faversham, UK) controlled by the Axon Imaging Workbench 2.2 software (Axon

Instruments, Union City, CA, USA) which was also used for image acquisition and analysis. Cells which responded with an increase in $\Delta F/F_0$ >0.2 (i.e. an increase in fluorescence by more than 20%, F_0 being the fluorescence level before thermal stimulation) during cooling were considered cold-sensitive.

We have investigated the effect of inflammatory mediators on CMS rat sensory neurones in culture. The change in intracellular calcium concentration ($[Ca^{2+}]_i$) with cooling was monitored and two parameters were measured for each cell: the ratio between the maximal fluorescence change during cooling and the initial fluorescence ($\Delta F/F_{0max}$, or 'amplitude'), and the temperature threshold for activation by cooling, defined as the temperature at which the fluorescence level exceeded the mean initial fluorescence by five standard deviations (calculated for the five fluorescence data points prior to the beginning of the stimulus). In a control experiment, a total of 14 CMS neurones were submitted to this cooling stimulus four consecutive times at 5 min interval and no significant changes occurred in amplitude or threshold. Inflammatory mediators were applied for 5 min between the second and the third stimulation by cooling. Some cells responded to the application of inflammatory agents with an increase in $[Ca^{2+}]_i$, and this response was also recorded. After the fourth cooling stimulus, (-)-menthol was applied at 100 μM and then the neurones were cooled in the presence of menthol, in order to assign menthol sensitivity. CMS neurones were considered not only those that responded to menthol at the base temperature, but also those in which the temperature threshold for activation by cooling was shifted to warmer temperatures in the presence of menthol. Finally, the TRPA1 agonist cinnamon aldehyde (200 μM, for 2 min) was applied at the base temperature of ~32°C. Figure 1 illustrates the experimental protocol. It shows a CMS neurone responding to the application of 10 μM bradykinin (BK), but also to 100 μM menthol (ME) and 200 μM cinnamon aldehyde (or cinnamaldehyde, CA).

The IncMix solution for DRG incubation contained (in mM): NaCl, 155; K_2HPO_4, 1.5; HEPES, 5.6; NaHEPES, 4.8; glucose, 5. The antibiotic gentamicin was added to 50 μg/ml.

The standard extracellular solution used in all experiments contained (in mM) NaCl, 140; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; Hepes, 10; NaOH, 4.55; glucose, 5 (pH 7.4 at 25°C).

Drugs were added from the following stock solutions: bradykinin (Sigma), 10 mM in H_2O; PGE_2 (Sigma), 10 mM in ethanol; histamine (Sigma), 10 mM in H_2O; menthol (Sigma), 200 mM in ethanol; cinnamon aldehyde (Sigma), 200 mM in ethanol; PMA (phorbol 12-myristate 13-acetate) (Sigma), 1 mM in DMSO; KT 5720 (Sigma), 1 mM in DMSO; 8-Br-cAMP (Sigma), 100 mM in 0.1 N NaOH; Ro-31-8220 (Calbiochem), 1 mM in DMSO. All drug dilutions were prepared on the day of the experiment and the menthol solution was renewed every 2 h because of evaporation. The vehicle used for dissolving the drugs was also present in the standard extracellular solution to prevent any effects induced by the solvent.

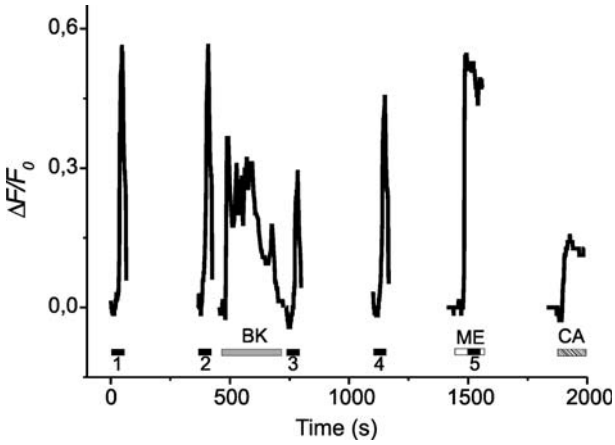

Fig. 1 Experimental protocol. An example of a CMS neurone submitted to five cold stimuli, bradykinin (BK), menthol (ME) and cinnamon aldehyde (CA) is shown. The *bars* underneath the traces represent the applications of cold stimuli (*black*), inflammatory mediators (*grey*), menthol (*white*) and cinnamon aldehyde (*hatched*). Note the reduction in the amplitude of the response to cooling after BK, and the partial recovery of the response at the fourth stimulation. This cell responded to the application of ME at the basal temperature and did not respond further to cooling in the presence of menthol. Other CMS neurones do not respond to the application of ME at basal temperature, but their response to cooling in ME is strongly sensitised

The data presented in this study consist mainly in statistical comparisons between the second and the third response to cooling (the third stimulus was applied after the treatment with inflammatory mediators) in terms of amplitude and threshold temperature. Data are presented as mean ± SEM. Two-tailed Student's paired *t* test was performed using the Origin 7 software (OriginLab Corporation, Northampton, MA, USA) and the χ^2 test was performed using Statistica 7.1 (StatSoft Inc, Tusla, OK, USA). A value of $P < 0.05$ was considered statistically significant.

Results

Cold- and menthol-sensitive neurones are activated by inflammatory mediators

The changes in $[Ca^{2+}]_i$ induced by the application of bradykinin (BK), prostaglandin E_2 (PGE_2) and histamine

(HA) (10 μM each) were recorded in cultured DRG neurones at 32°C, and these neurones were also tested for sensitivity to cold and menthol.

An interesting observation was that a remarkably high fraction of the CMS neurones responded to the application of BK, PGE_2 or HA at 32°C. Figure 2a shows examples of individual neurones activated by BK, PGE_2 and HA (from top to bottom) and Fig. 2b shows the response of the same neurones to the application of 100 μM menthol.

Of 24 CMS neurones, seven (29%) responded to the application of 10 μM BK at 32°C (Fig. 1). In contrast, of the remaining 137 DRG neurones imaged, only 17 (12%) were activated by BK, demonstrating a remarkable co-expression of the sensitivity to cold and menthol and to BK ($P < 0.05$, χ^2 test, Fig. 3a). Interestingly, six of the seven BK-sensitive CMS cells were also activated by cinnamaldehyde (CA), while only 9 of the 17 BK-insensitive CMS cells responded to CA. The response to cooling measured before the application of BK was smaller in BK-sensitive CMS neurones compared to the BK-insensitive CMS cells ($\Delta F/F_0$: 0.31 ± 0.02, $n = 7$, and 0.45 ± 0.04, $n = 17$, respectively, $P = 0.02$, Student's unpaired t test), while the temperature threshold was not significantly different.

Prostaglandin E_2 induced an increase in $[Ca^{2+}]_i$ in 11 of 23 CMS neurones (48%), while very few of the remaining cold- and menthol-insensitive neurones imaged responded to PGE_2 (18 of 198, 9%, $P < 0.001$ compared to CMS neurones, χ^2 test, Fig. 3a). Among CMS neurones, sensitivities to PGE_2 and CA were highly co-expressed: 10 of the 11 PGE_2-sensitive cells also responded to CA, while none of the 12 PGE_2-insensitive neurones was activated by it. Again, the responses to cooling recorded before application of PGE_2 were smaller in PGE_2-sensitive than in PGE_2-insensitive CMS neurones ($\Delta F/F_0$: 0.37 ± 0.04, $n = 11$,

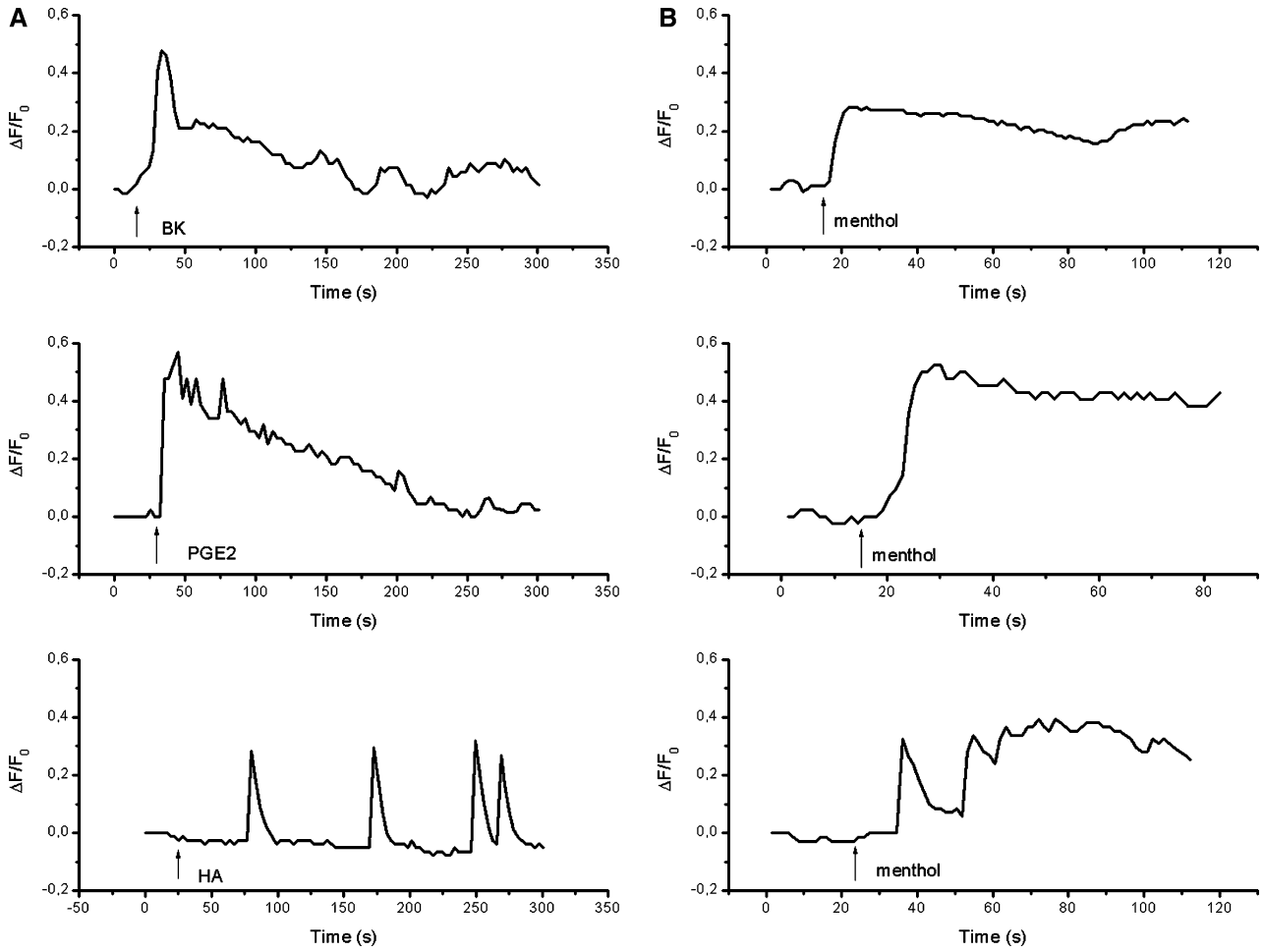

Fig. 2 Changes in intracellular calcium concentration induced by inflammatory agents and menthol in the same DRG neurones. **a** Example of fluorescence change induced by bradykinin (BK), prostaglandin E_2 (PGE_2) and histamine (HA) (from *top* to *bottom*) in cultured rat DRG neurones. **b** The same neurones as in **a** are activated by 100 μM menthol. The *arrows* in both parts indicate the times at which the chemical agents were applied (the application was continuous for the whole duration shown in the figure)

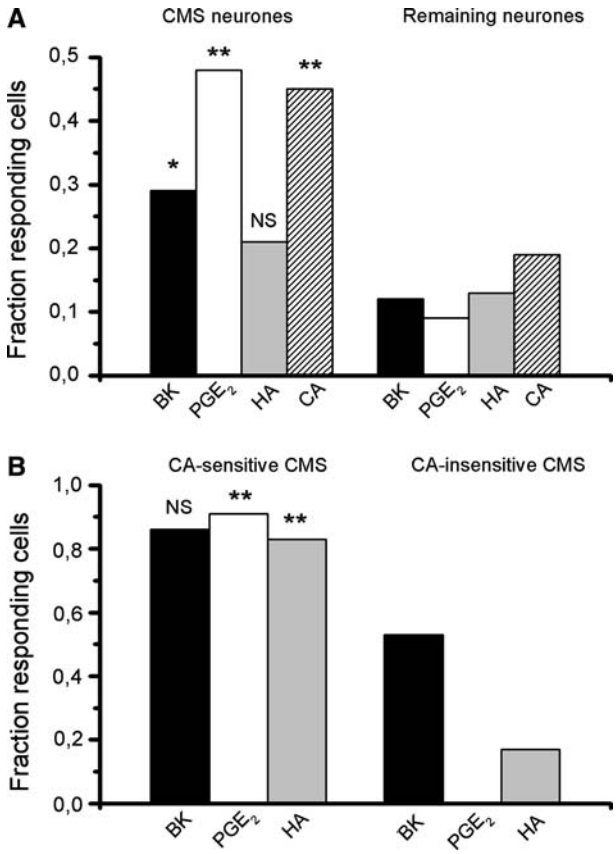

Fig. 3 Cold- and menthol-sensitive neurones express a remarkable sensitivity for inflammatory mediators. **a** The fractions of neurones responding to BK (*black columns*), PGE_2 (*white columns*), HA (*grey columns*) and CA (*hatched columns*) are represented for CMS neurones (*left*) and for the remaining neuronal population (*right*). The results of the statistical comparison of the fractions using the χ^2 test are given (*$P < 0.05$; **$P < 0.01$, *NS* nonsignificant). **b** The fractions of neurones responding to BK (*black columns*), PGE_2 (*white columns*) and HA (*grey columns*) are represented for CA-sensitive (*left*) and CA-insensitive (*right*) CMS neurones. The results of the statistical comparison of the fractions using the χ^2 test are given (*$P < 0.05$; **$P < 0.01$, *NS* nonsignificant)

compared to 0.52 ± 0.05, $n = 12$, $P = 0.03$). The temperature threshold was not significantly different.

Histamine activated 6 of 29 CMS neurones (21%). In four of these six neurones the response to histamine displayed such short bursts of calcium transients as shown in Fig. 2a, lower part. The reason for this behaviour is unclear. Of the remaining 184 neurones, 23 were also activated by HA (13%, not significantly different from CMS neurones, χ^2 test, Fig. 3a). Just like in the case of BK and PGE_2, five of the six HA-sensitive CMS neurones also responded to CA, while only four of the 23 HA-insensitive CMS cells were activated by it. There was no significant difference in the responses to cooling of the HA-sensitive and HA-insensitive CMS neurones.

Interestingly, sensitivity to the TRPA1 agonist cinnamaldehyde seems to be also more prominent in the CMS group (34/76, 45%), compared to the remaining neuronal population (96/516, 19%, $P < 0.001$, χ^2 test). The CA-sensitive CMS neurones had significantly smaller responses to cooling than the CA-insensitive CMS group (0.41 ± 0.02, $n = 34$, compared to 0.49 ± 0.02, $n = 42$, $P = 0.02$).

Our data indicate a strong sensitivity to inflammatory mediators among CMS neurones compared to the remaining DRG population (Fig. 3a). Moreover, two sub-populations could be identified within the CMS group: one with stronger responses to cooling and insensitive to inflammatory mediators and cinnamaldehyde, and another less responsive to cooling, but highly sensitive to inflammatory agents and cinnamaldehyde (Fig. 3b).

Inflammatory mediators bradykinin and prostaglandin E_2 inhibit the response to cooling in cold- and menthol-sensitive DRG neurones

In a total of 24 CMS neurones BK had no effect on the amplitude of the response to cooling, but shifted the temperature threshold for activation by cooling to colder temperatures (from 27.3 ± 0.7 to 26.3 ± 0.8 °C, $P = 0.02$, $n = 24$, Student's paired t test). The effects were more pronounced when the cells were divided into BK-sensitive (i.e. showing an increase in $[Ca^{2+}]_i$ during BK application) and BK-insensitive. Thus, BK-sensitive CMS neurones showed a decline in both amplitude ($\Delta F/F_0$ decreased from 0.36 ± 0.04 to 0.28 ± 0.03, $P = 0.04$, Figs. 1, 4a, b) and threshold temperature (from 25.7 ± 0.9 to 23.1 ± 0.8°C, $P = 0.008$, $n = 7$, Fig. 4a, b), while there was no significant effect on BK-insensitive CMS cells. The effects were partly reversed after 5 min wash with extracellular solution (Fig. 1).

In order to investigate the signalling pathway mediating the inhibition of CMS neurones by BK, the PKC activator PMA (phorbol 12-myristate 13-acetate) was applied at 1 μM for 5 min, between cold stimuli 2 and 3. Although there was no change in the amplitude of the response to cooling, the threshold temperature was strongly shifted to colder temperatures (from 28.5 ± 0.9 to 24.2 ± 1.3°C, $P = 0.002$, $n = 16$, Fig. 4a). Of the 16 CMS cells tested, 6 responded with an increase in $[Ca^{2+}]_i$ during application of PMA (PMA-responsive cells). In these six cells the effect of PMA was remarkably strong: the amplitude of the response was reduced by 60% ($\Delta F/F_0$ decreased from 0.33 ± 0.05 to 0.13 ± 0.09, $P = 0.02$, Fig. 4b) and the threshold became colder by almost 9°C (from

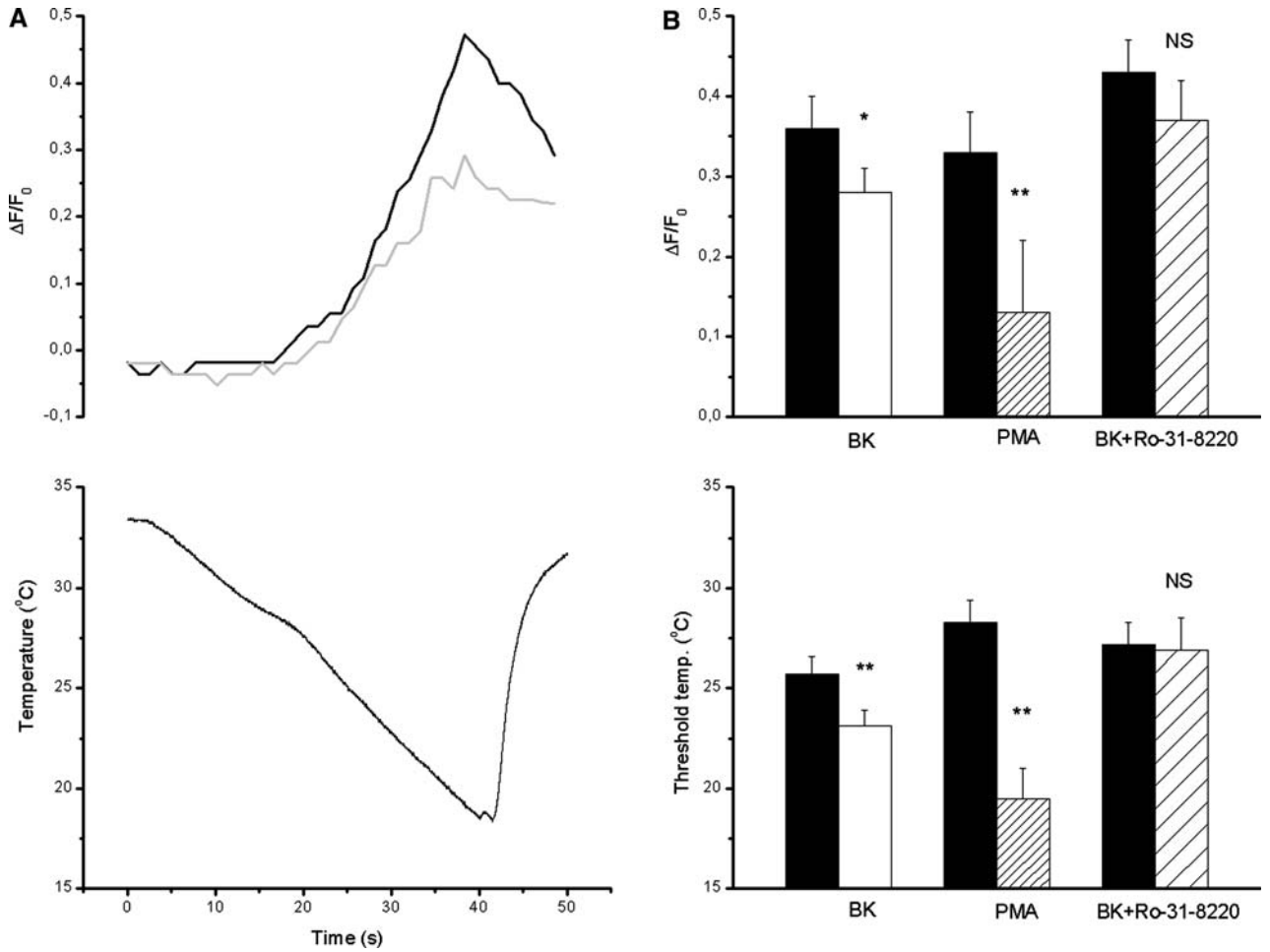

Fig. 4 Bradykinin inhibits cold responses in a subpopulation of cold- and menthol-sensitive neurones. **a**. *Upper part* the response to cooling in a CMS neurone before (*black*) and after the application of 10 μM BK (*grey*). This neurone is the same as the one showed in Fig. 2a, b, upper traces. *Lower part* the temperature stimulus measured with a T-type thermocouple placed where the cell had been is represented against time. Both upper and lower traces share the same time axis. **b** The effects of BK, PMA and Ro-31-8220 on the amplitudes (*upper part*) and thresholds (*lower part*) of the response to cooling in CMS neurones. Only the effects on CMS neurones responding to BK, PMA and BK in the presence of Ro-81-3220 are shown. *Black columns* represent the control values (before the application of BK, PMA or Ro-31-8220). The results of the statistical comparison using the paired Student's *t* test are also given (*$P < 0.05$; **$P < 0.01$, *NS* nonsignificant)

28.3 ± 1.1 to 19.5 ± 1.5 °C, $P = 0.004$, Fig. 4b). The remaining ten CMS neurones (PMA-unresponsive) were also inhibited by PMA, in that the threshold temperature was significantly reduced from 28.6 ± 1.2 to 26.5 ± 1.3°C ($P = 0.03$), while the amplitude of the response to cooling was not affected.

To further investigate the role of PKC in the BK-mediated inhibition of CMS neurones, BK was applied at 10 μM together with the selective PKC inhibitor Ro-31-8220 (1 μM). In a total of 24 CMS neurones BK had no effect on either the amplitude or the temperature threshold of the response to cooling in the presence of the PKC inhibitor. BK was still able to evoke an increase in $[Ca^{2+}]_i$ in the presence of Ro-31-8220 in eight CMS cells, possibly through PLC-mediated IP_3 synthesis and Ca^{2+} release from stores, but when these neurones were analysed separately there was no effect of BK on the response to cooling in these cells (Fig. 4b), nor in the remaining 16 neurones which did not respond to the application of BK.

The effect of PMA and the blocking effect of Ro-31-8220 suggest an involvement of PKC in the BK-mediated inhibition of CMS neurones.

PGE_2 induced a ~30% decrease in response amplitude in 23 CMS neurones (from $\Delta F/F_0$ 0.47 ± 0.04 to 0.34 ± 0.05, $P = 0.001$), accompanied by a shift in temperature threshold to lower temperatures (from 26.6 ± 0.6 to 24.1 ± 1.1°C, $P = 0.005$, $n = 23$). Of 23 CMS neurones, 11 responded to the application of 10 μM PGE_2 with an increase in $[Ca^{2+}]_i$, and were labelled PGE_2-responsive CMS cells. The inhibitory effect of PGE_2 was restricted to this group of CMS

neurones. Thus, in PGE_2-responsive CMS neurones we observed a pronounced decrease (of 55%) in response amplitude (from $\Delta F/F_0$ 0.40 ± 0.04 to 0.18 ± 0.04, $P = 0.001$, $n = 11$, Fig. 5a, b) and a strong reduction in temperature threshold for activation by cooling (from 26.2 ± 0.9 to 21.0 ± 1.4°C, $P = 0.002$, $n = 11$, Fig. 5a,b). There was no effect of PGE_2 in the remaining 12 unresponsive CMS cells.

The role of PKA in the inhibition of the responses to cooling in CMS neurones by PGE_2 was investigated using the membrane permeable cAMP analogue 8-bromo cyclic AMP (8-Br-cAMP). 8-Br-cAMP was applied for 5 min at 100 μM between cold stimuli 2 and 3 and its effect on the response to cold stimuli was monitored in 15 CMS neurones. Although there was no significant change in the response amplitude, the threshold temperature was decreased by ~3°C (from 28.6 ± 1.0 to 25.6 ± 1.3°C, $P = 0.01$, $n = 15$). The cAMP analogue evoked increases in $\Delta F/F_0$ in a subgroup of five CMS neurones, and its inhibitory effects were restricted to these cells: the amplitude was reduced ($\Delta F/F_0$ decreased from 0.34 ± 0.07 to 0.23 ± 0.09, $P = 0.006$, Fig. 5b) and the threshold was strongly shifted to colder temperatures (from 28.1 ± 2.6 to 20.7 ± 2.1°C, $P = 0.02$, Fig. 5b). The response to cooling was not affected by 8-Br-cAMP in the remaining ten neurones (unresponsive to 8-Br-cAMP).

The involvement of PKA in the PGE_2-mediated inhibition of CMS neurones was further investigated using the specific inhibitor KT5720. PGE_2 was applied

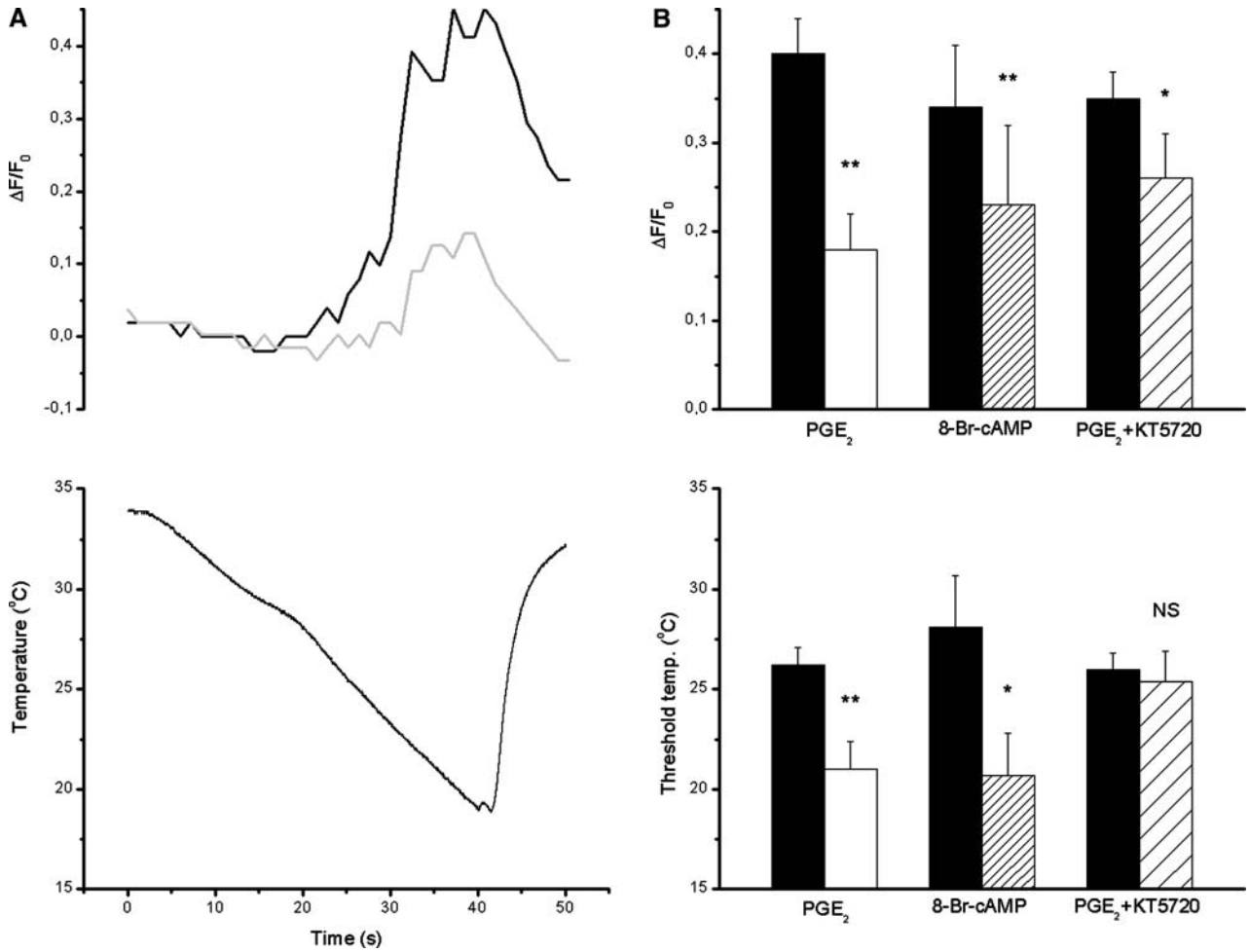

Fig. 5 Prostaglandin E_2 inhibits cold responses in a subpopulation of cold- and menthol-sensitive neurones. **a** *Upper part* the response to cooling in a CMS neurone before (*black*) and after the application of 10 μM PGE_2 (*grey*). This neurone is the same as the one showed in Fig. 2a, b, middle traces. *Lower part*: the temperature stimulus measured with a T-type thermocouple placed where the cell had been is represented against time. Both upper and lower traces share the same time axis. **b** The effects of PGE_2, 8-Br-cAMP and KT5720 on the amplitudes (*upper part*) and thresholds (*lower part*) of the response to cooling in CMS neurones. Only the effects on CMS neurones responding to PGE_2, 8-Br-cAMP and PGE2 in the presence of KT5720 are shown. *Black columns* represent the control values (before the application of PGE_2, 8-Br-cAMP or KT5720). The results of the statistical comparison using the paired Student's *t* test are also given (*$P < 0.05$; **$P < 0.01$, *NS* nonsignificant)

for 5 min at 10 μM together with 1 μM KT5720, between cold stimuli 2 and 3. The effects of PGE_2 on the responses to cooling in CMS neurones were reduced in the presence of KT5720. Although the amplitude was still decreased (from $\Delta F/F_0$ 0.45 ± 0.05 to 0.37 ± 0.06, $P = 0.02$, $n = 16$), there was no effect on the threshold temperature. PGE_2 was still able to evoke increases in $[Ca^{2+}]_i$ in a subgroup of CMS cells ($n = 7$) in the presence of the PKA inhibitor. When these cells were analysed separately, the effect was similar to that observed on the whole population of CMS neurones: the amplitude was reduced (from $\Delta F/F_0$ 0.35 ± 0.03 to 0.26 ± 0.05, $P = 0.03$, $n = 7$) but the threshold was unaffected (Fig. 5b). There was no effect of PGE_2 in the presence of KT5720 on the remaining nine neurones.

Histamine alone did not induce any change in the response to cooling in 29 CMS neurones, nor did it affect the response to cooling in a subgroup of six CMS neurones which responded with an increase in $[Ca^{2+}]_i$ during the application of HA.

Discussion

The aim of this study was to describe the effects of selected inflammatory mediators on the responses to cooling of cultured rat DRG neurones. In particular we focused our attention on CMS neurones, most likely expressing TRPM8, the cold and menthol receptor. It was recently shown that TRPM8 expressed in *Xenopus* oocytes is inhibited by PKC activators such as PDBu (phorbol-12, 13-dibutyrate) and PMA (phorbol 12-myristate, 13-acetate), as treatment with these drugs leads to a reduction in TRPM8-mediated responses to both menthol and cooling (Premkumar et al. 2005). The same authors described a PKC-mediated inhibition of menthol-induced calcium transients by bradykinin in adult rat DRG neurones. In another report, PMA treatment induced desensitisation of menthol-activated currents in TRPM8 expressing human embryonic kidney (HEK) cells (Abe et al. 2005). Our study complements and extends these findings by describing the effects of selected inflammatory mediators on native TRPM8 and identifying some intracellular factors modulating its response to cooling.

We have used real time microfluorimetry to record changes in $[Ca^{2+}]_i$ induced by cooling DRG neurones from ~32 to ~18°C. Repetitive stimulation at 5 min interval produced reproducible responses. Inflammatory mediators bradykinin (BK), prostaglandin E_2 (PGE_2) and histamine (HA) at 10 μM each were used to treat CMS DRG neurones cultured in the absence of NGF.

An interesting observation was that a remarkably high proportion of CMS neurones were directly activated by BK (30%) or PGE_2 (48%). In contrast only 12% of the remaining neuronal population was directly activated by BK, and 9% by PGE_2. This suggests a high degree of co-expression of TRPM8 and both the B2 and the EP1 receptors, which have been proposed to mediate the excitatory action of BK and PGE_2, respectively, in DRG neurones (Kasai et al. 1998; Sugiura et al. 2002; Nakayama et al. 2004). The difference in the level of expression of HA sensitivity between CMS cells (21%) and the remaining DRG neurones (13%) was not statistically significant. Interestingly, sensitivity to inflammatory mediators was highly co-expressed with sensitivity to the TRPA1 agonist cinnamaldehyde (CA) in CMS neurones. Although in situ hybrydization work suggested that TRPM8 and TRPA1 are not co-expressed in rat DRG (Story et al. 2003), a significant proportion of CMS neurones responded to CA in our cultures in the absence of added NGF. This is in good agreement with previous work from our laboratory (Babes et al. 2004), showing that ~30% of CMS neurones (very likely expressing TRPM8) are sensitive to the TRPA1 agonist allyl isothiocyanate (mustard oil). This suggests a high degree of functional co-expression of TRPM8 and TRPA1 in our DRG culture conditions. CMS neurones responsive to inflammatory agents and to CA had smaller responses to cooling (measured in naïve cells, before application of inflammatory mediators) compared to the remaining CMS cells.

The very low percentage of BK-responding, cold- and menthol-insensitive neurones (17/137, 12%) was rather unexpected, considering that quite a substantial fraction of these cells should be heat-sensitive nociceptors, which are known to express both B1 and B2 receptors for BK (Petersen et al. 1998a; Vellani et al. 2004). This may be due to the absence of added NGF in our cultures, as it was shown that NGF increases the expression of BK receptors (Petersen et al. 1998b; Kasai et al. 1998). Moreover, high expression of BK responsiveness was reported to occur after at least 1 day in culture (~75% of rat DRG neurones responded to BK after ~40 h in the presence of NGF, Petersen et al. 1998a), while our experiments were performed within 24 h after plating the cells.

While HA alone had no effect, BK and PGE_2 reversibly inhibited the response to cooling in subpopulations of CMS neurones. In the case of BK, the inhibition consisted in a decrease in response amplitude and a shift of the threshold to colder temperatures (i.e. stronger cooling was required to trigger the calcium response), and was restricted to the subpopulation of BK-responsive CMS cells (neurones in which BK

evoked an increase in $[Ca^{2+}]_i$). The effect of PGE_2 was similar and also restricted to PGE_2-responsive CMS neurones.

Another set of experiments addressed the issue of the signalling pathways mediating the observed inhibition of CMS neurones by BK and PGE_2. BK activates constitutively expressed B2 receptors in DRG neurones, followed by stimulation of PLC, PIP_2 cleavage and generation of membrane bound diacyl glycerol (DAG) and cytoplasmic inositol trisphosphate (IP_3). DAG liberation triggers PKCε translocation to the plasma membrane and its subsequent activation (Cesare et al. 1999). We decided to investigate the role of PKC in the inhibition of CMS neurones by BK. The PKC activator PMA mimicked the effects of BK and evoked calcium transients in 40% of CMS neurones. However, upon PMA treatment the response to cooling was significantly reduced not only in the subpopulation of PMA-responsive neurones, but also in the remaining 60% CMS neurones. This agrees well with the ubiquitous nature of PKC: while BK could only inhibit CMS neurones expressing the B2 receptor, PMA had the same effect on the whole CMS population. Co-application of BK together with the specific PKC inhibitor Ro-31–8220 completely abolished the inhibition of CMS neurones. This observation is in good agreement with the total reversal of BK-induced inhibition of calcium responses to menthol by the specific PKC inhibitor BIM (bisindolylmaleimide) in DRG neurones (Premkumar et al. 2005). Our results suggest that PKC is an important factor in mediating the acute inhibition of TRPM8 by BK in sensory neurones. However, a role of PIP_2 in the inhibition of native TRPM8 by BK cannot be excluded and must be further investigated (Liu and Qin 2005; Rohacs et al. 2005).

PGE_2 is known to sensitise DRG neurones and to induce hyperalgesia via cAMP production and PKA-mediated phosphorylation of cellular targets such as the capsaicin receptor TRPV1 (Lopshire and Nicol 1998; Bhave et al. 2002). We decided to investigate the role of the cAMP/PKA signalling pathway in the inhibition of CMS neurones by PGE_2. The membrane permeant cAMP analogue 8-Br-cAMP was able to reproduce the effects of PGE_2, but in contrast to PMA, the inhibition was restricted to the subpopulation of CMS neurones which responded to the bath application of 8-Br-cAMP, suggesting that an increase in intracellular calcium concentration may be required for the full inhibitory effect or that in some cells certain components of the signalling pathway are missing.

Co-application of PGE_2 together with the specific PKA inhibitor KT5720 resulted in a substantially diminished inhibition of CMS neurones. It was recently shown that the $G\alpha_S$ -protein can activate PKCε via cAMP production and activation of the guanine exchange factor Epac in a subpopulation of DRG neurones (Hucho et al. 2005). This may explain the incomplete block of the PGE_2 effect by the PKA inhibitor KT5720.

Histamine is thought to exert its excitatory action in sensory neurones by activating phospholipase A_2 followed by stimulation of lipoxygenase (Kim et al. 2004). The lack of an effect of HA on CMS neurones suggests that this pathway is less effective in modulating native TRPM8 channels.

It is important to mention that the inflammatory mediators used in this study are known to activate signalling pathways that modulate a variety of ionic conductances involved in controlling the excitability of sensory neurones. Combined with a putative inhibition of TRPM8, this modulation may contribute to the desensitisation of CMS neurones induced by BK and PGE_2.

A nociceptive function of a subgroup of capsaicin-responsive CMS neurones has already been proposed by Viana et al. 2002. Very recently, it was shown that this capsaicin-sensitive CMS group expresses other nociceptive features, such as ATP sensitivity, a sustained response to acid pH and expression of TTX-resistant sodium channels (Xing et al. 2006). Our results confirm the existence of two functionally distinct populations of CMS (very likely TRPM8-expressing) neurones, one of which is sensitive to pungent compounds and inflammatory agents and possibly serves a nociceptive role.

The response to cooling in this subpopulation is inhibited by acute application of the inflammatory mediators BK and PGE_2, and this effect is at least partly mediated by protein kinases A and C. Gentle cooling is known to alleviate the effects of inflammation and recent behavioural studies have shown that activation of TRPM8 antagonises capsaicin-induced nociception (Premkumar et al. 2005). Our results indicate that tolerance to cooling is increased by inflammatory mediators desensitizing cold receptors. This may enhance the activation of heat-sensitive nociceptors by inflammatory agents and increase the gain for inflammatory pain.

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