

In vivo evidence for a role of protein kinase C in peripheral nociceptive processing

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1 The present study was designed to characterize the nociceptive response induced by protein kinase C (PKC) peripheral activation and to investigate if this biochemical event is important for the nociceptive response induced by formaldehyde, and bradykinin (BK).

2 Intraplantar injection of phorbol-12,13-didecanoate (PDD; 0.01, 0.1 or 1 μ g), a PKC activator, but not of 4 α -PDD (inactive analogue), dose-dependently induced thermal hyperalgesia in rats. This response was not observed at the contralateral hindpaw. Intraplantar injection of PDD (0.01, 0.1 or 1 μ g) also induced mechanical allodynia. In mice, injection of PDD (0.1 or 1 μ g) into the dorsum of the hindpaw induced a spontaneous licking behaviour.

3 Intraplantar co-injection of chelerythrine (10 or 50 μ g), a PKC inhibitor, attenuated the thermal hyperalgesia induced by PDD (0.1 μ g) in rats.

4 The second phase of the nociceptive response induced by the injection of formaldehyde (0.92%, 20 μ l) into the dorsum of mice hindpaws was inhibited by ipsi-, but not contralateral, pre-treatment with chelerythrine (1 μ g).

5 Intraplantar injection of BK (10 μ g) induced mechanical allodynia in rats. Ipsi- but not contralateral injection of bisindolylmaleimide I (10 μ g), a PKC inhibitor, inhibited BK-induced mechanical allodynia.

6 In conclusion, this study demonstrates that PKC activation at peripheral tissues leads to the development of spontaneous nociceptive response, thermal hyperalgesia and mechanical allodynia. Most importantly, it also gives *in vivo* evidence that peripheral PKC activation is essential for the full establishment of the nociceptive response induced by two different inflammatory stimuli.

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Abbreviations: BK, bradykinin; CGRP, calcitonin gene-related peptide; DAG, diacylglycerol; DMSO, dimethylsulfoxide; DRG, dorsal root ganglion; IL, interleukin; IP₃, inositol triphosphate; i.pl., intraplantar; NK₁, neurokinin 1; NMDA, *N*-methyl-D-aspartic acid; PDD, phorbol-12,13-didecanoate; PGE₂, prostaglandin E₂; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol-12-myristate-13-acetate; s.c., subcutaneous; SP, substance P; TNF, tumour necrosis factor

Introduction

Protein kinase C (PKC), a serine/threonine kinase that exists in at least 11 isoforms (see Hofmann, 1997; Mochly-Rosen & Kauvar, 1998; Way *et al.*, 2000 for reviews), is an important regulator of various cellular functions such as growth, differentiation, neurotransmitter release and membrane excitability (Miller, 1986; Nishizuka, 1986; Kaczmarek, 1987). There are many studies describing the role of PKC in the nociceptive processing at the level of the spinal cord. Young *et al.* (1995) demonstrated that iontoporetic injection of chelerythrine, a PKC inhibitor, directly into the spinal cord, attenuates the hyperactivity of secondary neurons induced by mustard oil application onto the hindpaws of rats. Meller *et al.* (1996) showed that the thermal hyperalgesia induced by *N*-methyl-D-aspartic acid (NMDA) intrathecal injection is also inhibited by chelerythrine. A study conducted by

Malmberg *et al.* (1997) demonstrated that PKC γ knock-out mice do not develop hyperalgesia or allodynia as a result of sciatic nerve ligation. Also, these mice do not present neurochemical changes such as enhancement of neurokinin 1 (NK₁) receptor immunoreactivity or reduction of substance P (SP) immunoreactivity, alterations that usually develop after nerve ligation. Since the PKC γ isoform distribution is restricted to the spinal cord (Mori *et al.*, 1990; Malmberg *et al.*, 1997) and the route of injection of drugs in most studies was intrathecal, no mention can be done concerning the role of PKC in the process of stimulation or sensitization of the peripheral terminals of the primary afferent neurons, based on the studies cited above.

A few studies, however, clarify some of this issue, but mostly with electrophysiological data from preparations of dorsal root ganglion (DRG) neurons. Although the role of PKC in the processes of stimulation and sensitization of nociceptors is still poorly understood, it is likely that changes in neuronal function such as increase in calcium influx and inhibition of potassium currents take part in these phenotypic

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alterations. Both these changes can be elicited by intracellular microinjection of PKC activators (Deriemer *et al.*, 1985; Alkon *et al.*, 1986). Treatment with phorbol esters leads to nociceptor excitation (Leng *et al.*, 1996; Schepelmann *et al.*, 1993) as well as its sensitization to heat (Leng *et al.*, 1996) or mechanical stimulation (Schepelmann *et al.*, 1993). However, these results do not necessarily indicate that PKC activation is involved in the nociceptor stimulation or sensitization that results from the action of inflammatory mediators that participate in physiopathological responses.

An *in vitro* study demonstrated that ionic currents activated by heat in nociceptive neurons are facilitated by bradykinin (BK) and that the effect of this peptide is enhanced by phosphatase inhibitors, besides being mimicked by phorbol-12-myristate-13-acetate (PMA), a PKC activator (Cesare & McNaughton, 1996). Mizumura *et al.* (1997) also demonstrated that PKC is involved in the excitatory and facilitatory effects of BK on ionic currents in visceral nociceptors *in vitro*.

Nonetheless, a study by Khasar *et al.* (1999) addressed the question of PKC peripheral activation in nociceptive models by demonstrating that the thermal and mechanical hyperalgesia induced by intraplantar (i.pl.) injection of epinephrine and the acetic acid-induced writhing response are markedly attenuated in PKC ϵ knock-out mice. Moreover, in the same study, the authors showed that, in rats, the intradermal injection of a peptide that selectively inhibits PKC ϵ , attenuates the hyperalgesia induced by epinephrine, carrageenan and nerve growth factor and also the enhancement of tetrodotoxin-resistant sodium currents induced by epinephrine in DRG neurons.

In support to a involvement of PKC ϵ in the peripheral mechanisms of nociception, Cesare *et al.* (1999) showed that, among the five PKC isoforms present in DRG neurons (β I, β II, δ , ϵ and ζ), only the ϵ isoform is translocated, and consequently activated after BK treatment. In addition, the facilitation of ionic currents induced by heat, that follows BK treatment of the cells in culture, is attenuated by the same PKC ϵ inhibitor peptide used in the study of Khasar *et al.* (1999).

However, considering the relative lack of studies that investigated the role of PKC in the peripheral nociceptive transduction *in vivo*, and taking into account the data afforded by the studies of Khasar *et al.* (1999) and Cesare *et al.* (1999), we designed a study to extensively evaluate the behavioural effects induced by peripheral injection of a PKC activator, phorbol-12,13-didecanoate (PDD), in mice and rats. We have also investigated if PKC activation is essential for the full development of the nociceptive response to two important inflammatory stimuli, formaldehyde and BK, using the PKC inhibitors, chelerythrine or bisindolylmaleimide I.

Methods

Experimental animals

Male Swiss mice (25–45 g) and Wistar rats (250–350 g) were used. The animals were allowed to acclimatize in the experimental room for at least 3 days before the experiment. The experiments were carried out at an ambient temperature of 27°C, which corresponds to the thermoneutral zone for

rodents. Food and water were available *ad libitum*. Throughout the experiments, the animals were always carefully handled, so as to result in the least behavioural stress. Intraplantar injections were performed with the animal gently restrained in a soft piece of cloth, with the assistance of a second experimenter. All the experiments were approved by the Ethics Committee on Animal Experimentation (CETEA) of the Federal University of Minas Gerais.

Nociceptive models

Evaluation of thermal hyperalgesia in rats Paw withdrawal latency to a thermal stimulus was measured with a plantar test unit (model 7370, Ugo Basile, Italy) following i.pl. injection of PDD at different doses (0.01, 0.1 or 1 μ g) according to the method described by Hargreaves *et al.* (1988). Briefly, rats were kept in Perspex cells having a glass pane as a floor, and a source of infrared light was directed towards the hindpaw plantar surface. The latency for paw withdrawal from the radiant heat was automatically determined. At the beginning of the experiments the intensity of the infrared emission was adjusted so that the baseline value was close to 10 s. Cut-off time was 20 s. At each time point, the latencies were recorded twice at least 30 s apart in each animal and the average between the two measurements was considered for statistical analysis. After determining the baseline latency, the animals were divided into the various treatment groups so that the mean baseline values for all groups were similar. The latencies were measured 1, 3, 6, 10 and 20 h after PDD injection. For the protocol of reversal of PDD-induced thermal hyperalgesia by chelerythrine, the latency for paw withdrawal to radiant heat was measured 2 h after PDD/chelerythrine co-injection. A co-injection protocol was chosen to avoid a more extensive tissue damage that could have resulted if the animals had received two injections.

Evaluation of mechanical allodynia in rats Mechanical allodynia was measured with a 100 mN von Frey filament. Briefly, the rats were kept in Perspex boxes whose floor was a metal grid through which the filament was pressed onto the plantar surface of the hindpaws with the strength just necessary to cause the filament to bend for approximately 1 s. The number of withdrawal reflexes was determined in a trial of 10 touches for each rat. The model was used to determine if i.pl. injection of PDD could induce mechanical allodynia. Before injections, a basal response was determined and animals with a high rate (>3 responses for each trial of ten touches) were excluded from the experiment. Then the animals were distributed to their respective treatment groups in a manner that the mean baseline values were similar among the different groups. Different doses were used (0.01, 0.1 or 1 μ g, 50 μ l) and mechanical allodynia was tested 1, 3, 6, 10 and 20 h after PDD or vehicle injection.

The model was also used to test if BK (1 or 10 μ g, 50 μ l) could induce mechanical allodynia 0.5, 1 and 2 h after injection in rats. This protocol was followed by two other experiments in which we investigated if PKC activation is involved in the local mechanism of BK-induced mechanical allodynia. This was achieved by co-injecting BK and bisindolylmaleimide I, a PKC inhibitor, or injecting BK into the right hindpaw and bisindolylmaleimide I into the left

(contralateral) hindpaw in different groups of rats. These two protocols were used to (i) verify the possibility of an inhibition of BK-induced mechanical allodynia by a PKC inhibitor and (ii) rule out that the inhibition, in case it is observed, results from systemic distribution rather than a local action of the drug.

Evaluation of spontaneous nociceptive behaviour in mice
Spontaneous nociceptive behaviour was assessed after subcutaneous (s.c.) injection of 20 μ l of a solution containing 0.1 or 1 μ g of PDD in mice. Similarly to the model described by Hunskaar & Hole (1987) for formalin-induced nociceptive behaviour, the model consisted of determining the time the animals spent licking the injected hindpaw. The period of 60 to 120 min was chosen because preliminary experiments in our laboratory demonstrated that the nociceptive response was most intense at this interval.

In two different protocols, it was tested if PKC peripheral activation was necessary for the expression of the nociceptive response in the formaldehyde model (formaldehyde 0.92%, 20 μ l, s.c.) by injecting 20 μ l of a solution containing 0.1 or 1 μ g of chelerythrine into the hindpaw, 30 min before formaldehyde injection. Licking behaviour was determined at the first and second phases of the nociceptive response to formaldehyde, i.e. 0–5 and 15–30 min after injection (Vaz *et al.*, 1996). Similarly to what was done for the mechanical allodynia induced by BK, in one of the protocols the PKC inhibitor was injected at the paw contralateral to that treated with the inflammatory stimulus. As in the latter case, this was done to test if the inhibitory effect was a consequence of systemic distribution of the drug.

Drugs

PDD (Sigma, U.S.A.), 4 α -PDD (Sigma, U.S.A.), chelerythrine chloride (Sigma, U.S.A.), bisindolylmaleimide I hydrochloride (Sigma, U.S.A.), formaldehyde (Ecibra, Brazil), bradykinin acetate salt (Sigma, U.S.A.) and dimethylsulphoxide (DMSO, Merck, Brazil) were used. Stock solutions of PDD and 4 α -PDD were obtained by diluting them in DMSO 100%. BK solutions were prepared in sterile saline. Solutions of PDD, 4 α -PDD and BK were stored in a -70°C freezer. Chelerythrine stock solution was prepared in DMSO 50% in sterile saline and kept in a -20°C freezer. Bisindolylmaleimide I stock solution was prepared in DMSO 100% and stored at -20°C . Further dilutions of all drugs were done with sterile saline 0.9%. All drugs, except formaldehyde, were kept on ice until used. The concentration of DMSO in the vehicle used in each experiment depended on the doses of the drug used. When more than one dose was used for a drug, the concentration of DMSO in the vehicle corresponded to that present in the solution used to inject the highest dose of the drug. Adjustments in the concentration of solutions for the co-injection protocols were performed as if the drugs were delivered separately in 50 μ l, resulting in a final volume of 100 μ l.

Statistical analysis

The results were expressed as mean \pm s.e.mean and analysed by one-way ANOVA. Statistical difference was confirmed by the Newman–Keuls *post hoc* test and inferred at $P < 0.05$.

Results

Figure 1a shows that i.pl. injection of PDD (0.01, 0.1 or 1 μ g) induced a long-lasting thermal hyperalgesia in rats, that was present between 1 and 10 h after injection. When paw

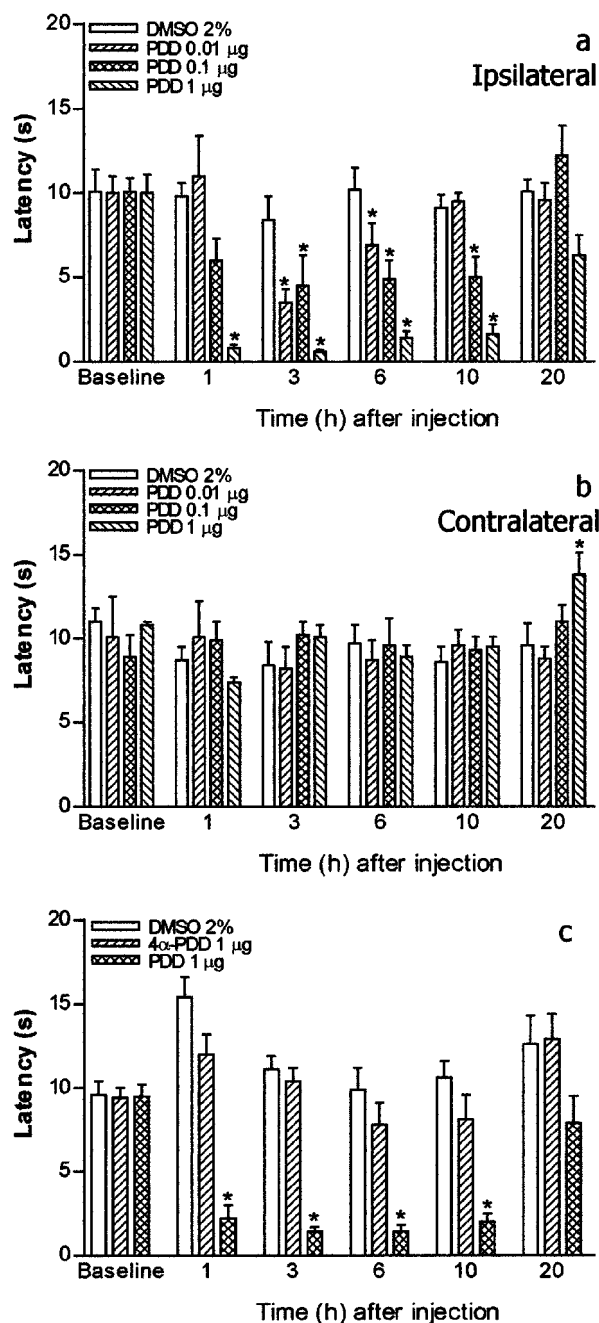


Figure 1 Effect of i.pl. PDD or 4 α -PDD injection on paw withdrawal latencies to radiant heat in rats. Animals received an injection of PDD, 4 α -PDD or vehicle after measurement of baseline values and were tested according to the model of thermal hyperalgesia. Time course of withdrawal latency is shown for the ipsilateral (a) and contralateral (b) paws after injection of different doses of PDD (0.01, 0.1 or 1 μ g, 50 μ l, i.pl.). (c) indicates the time course of thermal hyperalgesia induced by PDD (1 μ g, 50 μ l, i.pl.), but not 4 α -PDD (1 μ g, 50 μ l, i.pl.). * $P < 0.05$ compared with the vehicle-treated group, tested by ANOVA followed by Newman–Keuls *post hoc* test. Data are expressed as mean \pm s.e.mean ($n = 5-6$).

withdrawal latencies were evaluated 20 h after the injection, the values did not differ from those of the control group. Thermal hyperalgesia did not occur at the contralateral paw over the time period observed (Figure 1b). In addition, i.pl. injection of a PDD inactive analogue, 4 α -PDD (1 μ g), induced no thermal hyperalgesia at any time point tested (Figure 1c).

Von Frey filaments were employed to investigate if peripheral activation of PKC leads to mechanical allodynia. As shown in Figure 2, i.pl. injection of PDD (0.01, 0.1, or 1 μ g) in rats induced a marked and sustained dose-dependent mechanical allodynia in the injected paw.

Figure 3 shows that subcutaneous (s.c.) injection of PDD (0.1 or 1 μ g) into mice hindpaws dose-dependently induced a spontaneous licking behaviour which was statistically significant for the dose of 1 μ g. As described in the Methods section, licking behaviour was determined between 60 and 120 min after PDD or vehicle injection.

Figure 4 shows that both doses chelerythrine (10 or 50 μ g, i.pl.), when co-injected with PDD (0.1 μ g, i.pl.), completely abolished PDD-induced thermal hyperalgesia when this response was measured 2 h after PDD injection. The highest dose of chelerythrine *per se* did not alter thermal withdrawal latencies.

To investigate the involvement of PKC in the peripheral processing of the nociceptive response in the formaldehyde model, we pre-treated mice with chelerythrine (0.1 or 1 μ g, s.c., -30 min) at the same paw later injected with formaldehyde (0.92%, 20 μ l, s.c.). Figure 5a shows that chelerythrine dose-dependently inhibited the second phase of formaldehyde-induced licking behaviour. The effect of chelerythrine was demonstrated to be dependent on local, but not systemic PKC inhibition, because when the same dose of this drug was injected into the contralateral paw, no inhibition of formaldehyde-induced nociception was observed (Figure 5b).

Mechanical allodynia was also demonstrated to occur after i.pl. injection of BK (1 and 10 μ g) as shown in Figure 6a. Interestingly, BK-induced mechanical allodynia likely involves PKC activation at peripheral tissue because bisindo-

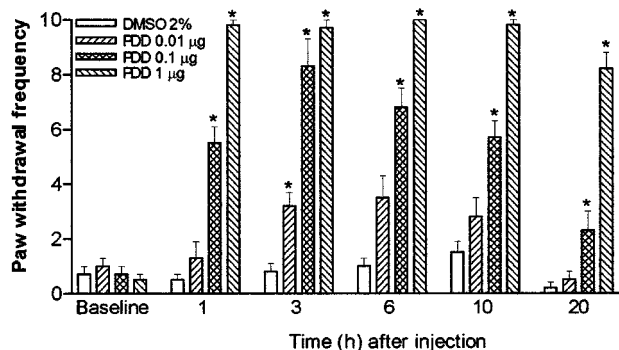


Figure 2 Effect of i.pl. PDD injection on paw withdrawal frequency to stimulation with a von Frey filament (100 mN) in rats. Animals received an injection of PDD (0.01, 0.1 or 1 μ g, 50 μ l, i.pl.) or vehicle after measurement of baseline values and were tested according to the model of mechanical allodynia. The number of touches per trial was 10. * $P < 0.05$ compared with the vehicle-treated group, tested by ANOVA followed by Newman–Keuls *post-hoc* test. Data are expressed as mean \pm s.e.mean ($n = 6$).

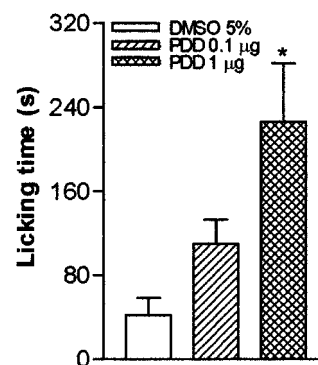


Figure 3 Paw licking behaviour induced by s.c. injection of PDD in mice. Animals received an injection of PDD (0.1 or 1 μ g, 20 μ l, s.c.) into the dorsum of the hindpaw and the time they spent licking the injected hindpaw was determined between 1 and 2 h later. * $P < 0.05$ compared with the vehicle-treated group tested by ANOVA followed by Newman–Keuls *post-hoc* test. Data are expressed as mean \pm s.e.mean ($n = 9$).

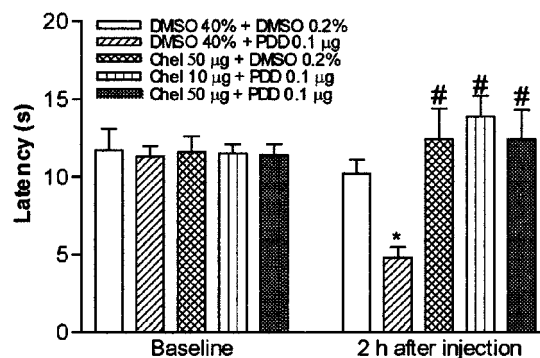


Figure 4 Effect of chelerythrine co-injection on PDD-induced thermal hyperalgesia in rats. Animals received injection of PDD (0.1 μ g), chelerythrine (10 or 50 μ g) and/or their respective vehicles, as shown in the figure and were tested 2 h later. Drugs or vehicle were collected into the same syringe in a volume of 50 μ l each, resulting in a final volume of 100 μ l, that was injected by the i.pl. route. * and # $P < 0.05$ compared with the groups treated with vehicle and DMSO 40% + PDD 0.1 μ g, respectively, tested by ANOVA followed by Newman–Keuls *post-hoc* test. Data are expressed as mean \pm s.e.mean ($n = 5-6$).

ylmaleimide I (1 or 10 μ g, i.pl.), when co-injected with BK, dose-dependently inhibited BK-induced mechanical allodynia (Figure 6b), an effect that was not observed when this PKC inhibitor was injected at the contralateral paw (Figure 6c).

Discussion

The present study broadly characterizes the effects of peripheral injection of a PKC activator, PDD, indicating that the local activation of this enzyme leads to thermal hyperalgesia, mechanical allodynia and spontaneous nociceptive licking behaviour. More interestingly, the study clearly demonstrates the involvement of PKC in the peripheral transduction of bradykinin-induced mechanical allodynia and

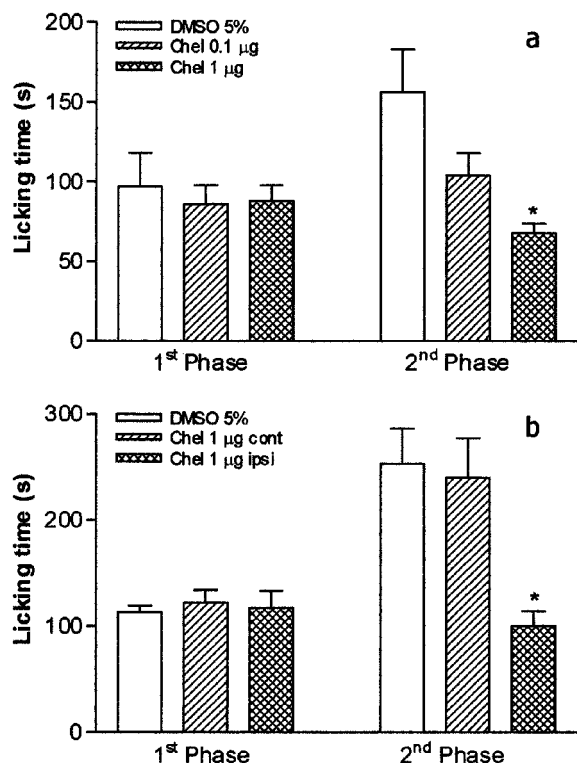


Figure 5 Effect of chelerythrine pre-treatment on the licking behaviour induced by formaldehyde injection into mice hindpaws. In (a) animals received an injection of chelerythrine (chel, 0.1 or 1 µg, 10 µL, s.c.) or vehicle into the dorsum of the hindpaw that was treated 15 min later with formaldehyde (0.92%, 20 µL, s.c.). In (b) chelerythrine (chel, 1 µg, 10 µL, s.c.) was injected at either the contralateral (cont) or ipsilateral (ipsi) hindpaw 15 min before the injection of formaldehyde (0.92%, 20 µL, s.c.). Licking behaviour was determined during the first (0–5 min) and second (15–30 min) phases of formaldehyde-induced nociceptive response. * $P < 0.05$ compared with the vehicle-treated group, tested by ANOVA followed by Newman–Keuls *post-hoc* test. Data are expressed as mean \pm s.e.mean ($n = 9$).

also of the second phase of the nociceptive response induced by formaldehyde, supporting the hypothesis that peripheral PKC activation might be a point of convergence in the signal transduction underlying various painful conditions.

We initially observed that peripheral injection of PDD induced a long-lasting thermal hyperalgesia. Our data suggest that this effect does not represent a nonspecific algogenic action, but rather results from alterations in the function of nociceptors and/or other cells at the site of injection that are dependent on PKC activation. This is highlighted by the fact that i.pl. injection of a PDD inactive analogue, 4 α -PDD, did not alter thermal withdrawal latencies. Also confirming the specificity of this response is the result demonstrating an inhibition of PDD-induced thermal hyperalgesia by chelerythrine, a PKC inhibitor. PDD peripheral injection also induced a mechanical allodynia, indicating that PKC activation sensitizes nociceptors to both thermal and mechanical stimuli.

How could PKC activation, under these circumstances, lead to a facilitation of the nociceptive response, as observed in the models of thermal hyperalgesia and mechanical allodynia? Two distinct actions might account for this. One

would depend on direct PKC activation in nociceptors. The other would be an indirect non-neuronal action that could rely on cells surrounding the injection site, with likely special participation of cells involved in the inflammatory response. Both hypotheses will be discussed as follows. However, which of these is more important for the effects observed after PDD peripheral injection remains to be determined and is beyond the scope of this study.

As it has been discussed earlier, treatment with phorbol esters increases the ongoing activity (Leng *et al.*, 1996; Schepelmann *et al.*, 1993) and sensitizes polymodal receptors to thermal (Leng *et al.*, 1996) and mechanical stimuli (Schepelmann *et al.*, 1993). These observations are in favour of a direct action of PDD on primary afferent fibres in our models of thermal hyperalgesia and mechanical allodynia. This is further supported by the demonstration that phorbol ester treatment of isolated rat skin enhances the heat-induced release of calcitonin gene-related peptide (CGRP), probably from the terminals of primary afferent fibres (Kessler *et al.*, 1999). Some studies indicate a pronociceptive effect of CGRP (Zhang *et al.*, 2001; Bennett *et al.*, 2000), and it is known that this peptide is often, if not always, co-released with SP (Frayer *et al.*, 1999), a potent hyperalgesic tachykinin (Nakamura-Craig & Smith, 1989). Based on this evidence, we propose that PDD injection leads to a facilitation of the nociceptive response by directly modulating the firing rate of sensory neurones, through mechanisms probably dependent on ion channel function (Alkon *et al.*, 1986; DeRiemer *et al.*, 1985), and also by causing them to release pronociceptive mediators that could act to sensitize their own terminals.

Nonetheless, evidence also exists to support a non-neuronal mechanism for PDD-induced nociceptive responses. PKC has been implicated in the expression of inducible nitric oxide synthase in murine cell lines after treatment with endotoxin (St-Denis *et al.*, 1998; Paul *et al.*, 1995) or interferon- γ (Momose *et al.*, 2000; Paul *et al.*, 1995). The production of the pro-inflammatory cytokines interleukin-1, IL-1, (St-Denis *et al.*, 1998), interleukin-6, IL-6, (Yamaki & Ohuchi, 1999) and tumour necrosis factor- α , TNF- α , (St-Denis *et al.*, 1998; Corsini *et al.*, 1999; Baumgartner *et al.*, 1996) by macrophages has also been demonstrated to be dependent on PKC activation. Moreover, Jacobson *et al.* (1995) have shown that mouse peritoneal macrophages produce prostaglandin E₂ (PGE₂) after PMA treatment, an effect inhibited by Gö 6850, a PKC inhibitor. Considering these data, we propose that peripheral injection of PDD besides exerting direct actions on nociceptors, could have induced the production of nitric oxide, IL-1, IL-6, TNF- α and/or PGE₂ by resident cells, with all or some of these inflammatory mediators leading to the establishment of an inflammatory process associated with a facilitation of nociceptive responses observed in our models. It is assumed that some of the mechanisms thought to underlie the sensitization to nociceptive stimuli would also result in the expression of a spontaneous nociceptive response, noted as an increase in paw licking behaviour. With the results described above, we are the first to fully characterize the nociceptive response to peripheral PKC activation by a phorbol ester.

We also observed that i.pl. injection of BK induced a mechanical allodynia in rats. A significant part of BK effect is thought to be dependent on direct activation of B₂ receptors

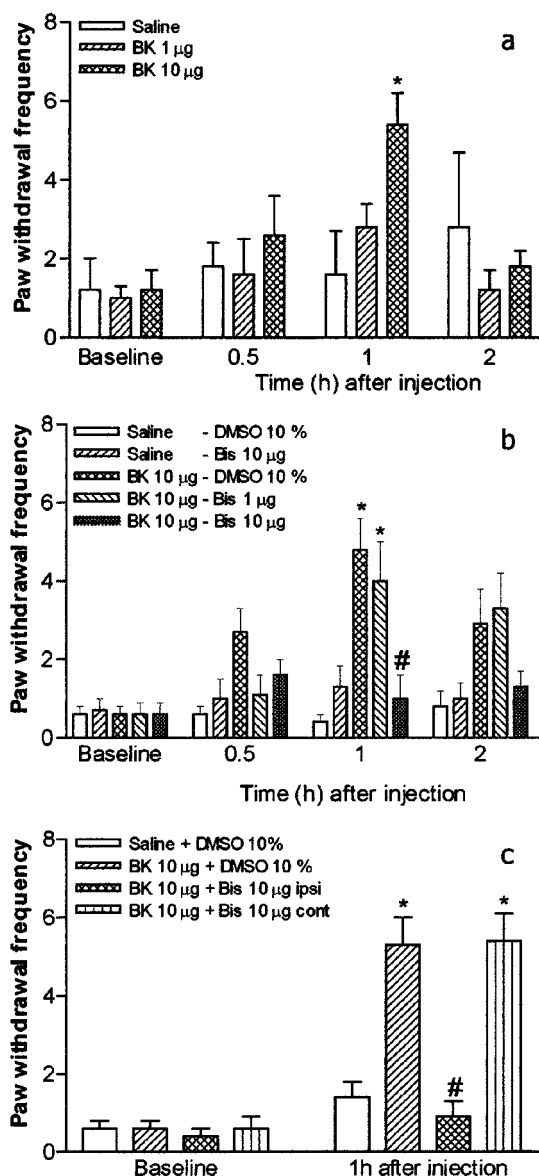


Figure 6 Mechanical allodynia induced by BK and its inhibition by bisindolylmaleimide I in rats. A von Frey filament of 100 mN was pressed 10 times per trial onto the plantar surface of each animal hindpaw to determine baseline response rates. Drugs were injected by the i.pl. route. In (b) and (c), drugs or vehicle were collected into the same syringe in a volume of 50 µl each, resulting in a final volume of 100 µl. (a) Time course of mechanical allodynia induced by BK (1 or 10 µg, 50 µl, i.pl.). (b) Effect of bisindolylmaleimide I (Bis, 1 or 10 µg) on the mechanical allodynia induced by BK (10 µg). (c) Effect of ipsi- or contralateral treatment with bisindolylmaleimide I (Bis, 10 µg) on the mechanical allodynia induced by BK (10 µg). * and # $P < 0.05$ compared with the groups treated with vehicle and BK 10 µg + DMSO 10%, respectively, tested by ANOVA followed by Newman–Keuls *post-hoc* test. Data are expressed as mean \pm s.e.mean ($n = 4-8$).

on the terminals of sensory neurones. This receptor has been shown to be present on DRG neurons (Davis *et al.*, 1996; Steranka *et al.*, 1988) and to mediate BK-induced hyperalgesia (Tonussi & Ferreira, 1997). In an electrophysiological study, Dray *et al.* (1992) have shown that the activation of nociceptors in a rat spinal cord-tail preparation by BK likely occurs through activation of intracellular signalling systems

consistent with the activation of PKC-dependent mechanisms. Similar results have been shown by McGuirk & Dolphin (1992) in rat sensory neurones. These data are in accordance with our observation that co-injection of bisindolylmaleimide I with BK abolished the mechanical allodynia induced by this inflammatory mediator. However, with the protocol used, it can not be stated that the bisindolylmaleimide I effect solely depends on the inhibition of PKC in sensory neurones. Instead, some of this effect may have arisen from the inhibition of BK effects on other cells such as macrophages, where it has been shown to induce the release of cytokines (Tiffany & Burch, 1989). Nevertheless, to our knowledge, this is the first report showing that bradykinin-induced allodynia is abolished by peripheral PKC inhibition.

We have also shown that pre-treatment with chelerythrine, a PKC inhibitor, at the hindpaw inhibited the second phase of formaldehyde-induced nociceptive behaviour in mice. Inhibition of nociceptive parameters has been shown after intrathecal injection of PKC inhibitors in a number of models, including the formaldehyde model (Yashpal *et al.*, 1995; Meller *et al.*, 1996; Hua *et al.*, 1999). In these studies, the inhibition was attributed to an impairment of PKC-induced facilitation of nociceptive transmission at the level of the spinal cord. In the present study, however, the inhibition of formaldehyde-induced nociceptive behaviour by chelerythrine could not be explained by the same mechanism, i.e., a spinal action, because a reduction in licking behaviour was only observed when the PKC inhibitor was injected into the same hindpaw treated with formaldehyde. An inhibition after injection into any hindpaw could be expected if the chelerythrine effect was dependent on systemic, especially spinal, PKC inhibition. However, this was not observed, from which we conclude that the effect of the inhibitor was local and not systemic.

Production and/or release of several mediators may account for the nociceptive response in the formaldehyde model. These include BK acting on B_2 receptors (Corrêa & Calixto, 1993), histamine acting on H_1 receptors (Mobarakeh *et al.*, 2000), 5-hydroxytryptamine acting on 5-HT $_{2A}$ receptors, among other subtypes (Doak & Sawynok, 1997; Abbott *et al.*, 1997), norepinephrine acting on α_{1A} adrenoreceptors (Hong & Abbott, 1996) and tachykinins acting on NK $_1$, NK $_2$ and NK $_3$ receptors (Santos & Calixto, 1997). Interestingly, many of the receptors with which these mediators interact were demonstrated to be coupled to the phospholipase C/diacylglycerol/inositol triphosphate (PLC/DAG/IP $_3$) signalling system, the cascade of intracellular events needed for activation of many PKC isoenzymes. This has been shown for B_2 (Challiss *et al.*, 1991; Plevin & Boarder, 1988), H_1 (Sipma *et al.*, 1996; Rugolo *et al.*, 1996), 5-HT $_{2A}$ (Xu *et al.*, 1996; Goppelt-Struebe & Stroebel, 1998), α_1 (Taguchi *et al.*, 1998; Williams *et al.*, 1998; Macrez-Leprêtre *et al.*, 1997) and NK $_1$ (Roush & Kwatra, 1998; Fukuhara *et al.*, 1998) receptors in a number of different cell types. The release of inflammatory mediators that act through the PLC/DAG/IP $_3$ signalling system is consistent with the chelerythrine inhibition of the second phase of formaldehyde-induced nociceptive response reported in this study. This effect may result from the impairment of PKC activation, an event downstream to the binding of the inflammatory mediators to their receptors. Moreover, the realization that peripheral PKC inhibition by two different

inhibitors attenuated the response to two different algogenic substances, i.e. formaldehyde and bradykinin, and in different animal species reinforces the assumption that PKC activation in peripheral tissues is a common event in nature that contributes to the establishment of the nociceptive response.

The inhibitory effect of bisindolylmaleimide I and chelerythrine on the mechanical allodynia induced by BK and the second phase of the nociceptive response induced by formaldehyde, respectively, indicates that the peripheral activation of PKC may be important for the full development of an immediate nociceptive response. However, the role of peripheral PKC in prolonged hyperalgesia or allodynia, as seen in experimental models like adjuvant-induced arthritis, nerve ligation or diabetic neuropathy, is unclear and further investigation is justified.

There has been a growing interest in the study of the peripheral involvement of PKC in the development of inflammatory processes. In this sense, it has been shown that the PKC ϵ isoform translocates to the membrane in response to BK in sensory neurones and probably mediates BK-induced heat sensitization in these neurones (Cesare *et al.*, 1999). In addition, Khasar *et al.* (1999) have shown that the mechanical allodynia induced by epinephrine or carrageenan can be inhibited by intradermal treatment with both bisindolylmaleimide I or a selective peptide inhibitor of PKC ϵ . Recently, the mechanical hyperalgesia associated with alcoholic neuropathy (Dina *et al.*, 2000) and also the prolonged mechanical hyperalgesia induced by carrageenan (Aley *et al.*, 2000) have also been attributed to PKC ϵ

activation in primary afferent fibres. All this evidence converges to the hypothesis that PKC activation may be an important event leading to the establishment of painful conditions, and that this enzyme may be an attractive drug target. However, an obvious demonstration in favour of this hypothesis was lacking, i.e., PKC activation *in vivo* should lead to the expression of a nociceptive response. Here we show this by plainly characterizing the thermal hyperalgesia, the mechanical allodynia and also the spontaneous nociceptive response resultant from PKC peripheral activation. Besides that, we clearly demonstrated that PKC local inhibition attenuates BK-induced mechanical allodynia and also the second phase of formaldehyde-induced licking behaviour, effects that were not inhibited by the same doses of PKC inhibitors injected into the contralateral hindpaw. In conclusion, the present study provides evidence for the involvement of PKC in peripheral nociceptive transduction, a fact that reaffirms the interest in the development of PKC inhibitors to be used in the management of pain.

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