BJP British Journal of Pharmacology

DOI:10.1111/bph.12488
www.brjpharmacol.org

RESEARCH PAPER

Peripheral interactions between cannabinoid and opioid systems contribute to the antinociceptive effect of crotalphine

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Keywords
crotalphine; antinociception; endogenous opioid release; dynorphin A; CB_2 cannabinoid receptors; cannabinoid–opioid interaction

Received
16 August 2013
Revised
17 September 2013
Accepted
5 October 2013

BACKGROUND AND PURPOSE
Crotalphine is an antinociceptive peptide that, despite its opioid-like activity, does not induce some of the characteristic side effects of opioids, and its amino acid sequence has no homology to any known opioid peptide. Here, we evaluated the involvement of the peripheral cannabinoid system in the crotalphine effect and its interaction with the opioid system.

EXPERIMENTAL APPROACH
Hyperalgesia was evaluated using the rat paw pressure test. Involvement of the cannabinoid system was determined using a selective cannabinoid receptor antagonist. Cannabinoid and opioid receptor activation were evaluated in paw slices by immunofluorescence assays using conformation state-sensitive antibodies. The release of endogenous opioid peptides from skin tissue was measured using a commercial enzyme immunoassay (EIA).

KEY RESULTS
Both p.o. (0.008–1.0 $\mu g \cdot kg^{-1}$) and intraplantar (0.0006 µg per paw) administration of crotalphine induced antinociception in PGE_2-induced hyperalgesia. Antinociception by p.o. crotalphine (1 $\mu g \cdot kg^{-1}$) was blocked by AM630 (50 µg per paw), a CB_2 receptor antagonist, and by antiserum anti-dynorphin A (1 µg per paw). Immunoassay studies confirmed that crotalphine increased the activation of both κ-opioid (51.7%) and CB_2 (28.5%) receptors in paw tissue. The local release of dynorphin A from paw skin was confirmed by *in vitro* EIA and blocked by AM630.

CONCLUSIONS AND IMPLICATIONS
Crotalphine-induced antinociception involves peripheral CB_2 cannabinoid receptors and local release of dynorphin A, which is dependent on CB_2 receptor activation. These results enhance our understanding of the mechanisms involved in the peripheral effect of crotalphine, as well as the interaction between the opioid and cannabinoid systems.

Abbreviations
AM1241, (3-iodo-5-nitrophenyl)-[1-[(1-methylpiperidin-2-yl)methyl]indol-3-yl]- methanone; AM630, [6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)-methanone (also known as iodopravadoline); EIA, enzyme immunoassay; i.pl., intraplantar

Introduction

Crotalphine is a 14 amino acid synthetic peptide that has recently been described as a new compound with an antinociceptive effect (Gutierrez *et al.*, 2008; Konno *et al.*, 2008). Its sequence is based on the structure of the natural analgesic factor isolated from the venom of the South American rattlesnake *Crotalus durissus terrificus* (Konno *et al.*, 2008). Previous work from our group demonstrated that crotalphine, when administered in low doses by the p.o., i.v. or intraplantar (i.pl.) routes, induced potent antinociceptive effects that lasted for 5 and 3 days in acute (Konno *et al.*, 2008) and chronic (Gutierrez *et al.*, 2008) pain models respectively. Additionally, this antinociceptive effect was blocked by i.pl. pretreatment with selective antagonists of either the κ (acute pain models) (Konno *et al.*, 2008) or κ and δ (chronic constriction injury of rat sciatic nerve) (Gutierrez *et al.*, 2012) opioid receptors, suggesting the involvement of peripheral opioid receptors in crotalphine's effect. Despite its opioid-like activity, crotalphine does not manifest some of the side effects that are characteristics of opioid drugs such as tolerance (Gutierrez *et al.*, 2008) and constipation (G.A. Rae, unpubl. data), even after prolonged treatment. Also, its amino acid sequence displays no homology to any known opioid peptide (Konno *et al.*, 2008), suggesting that opioid receptors might not be the target of crotalphine.

A significant interaction between endogenous opioid and cannabinoid systems in pain management (Cichewicz, 2004; Anand *et al.*, 2009) has been reported in the literature. Although opioids and cannabinoids bind to distinct receptors, it appears that one system may potentiate the other, which suggests that these two systems may operate synergistically (Cichewicz, 2004). Moreover, behavioural and molecular studies have demonstrated that cannabinoids induce the release of endogenous opioids just as opioids may induce the release of endocannabinoids (Ibrahim *et al.*, 2005; Welch, 2009). Furthermore, these studies have suggested the existence of direct receptor–receptor interactions as well as interactions in their intracellular signalling pathways when cannabinoid and opioid receptors are co-expressed in the same cells (Desroches and Beaulieu, 2010; Parolaro *et al.*, 2011).

The aim of the present study was to characterize the mechanisms involved in the antinociceptive effect of crotalphine by investigating the possible involvement of CB_2 cannabinoid receptors as well as the possible interaction of these receptors with the opioid system. Our results indicate that peripheral CB_2 cannabinoid receptors are involved in the antinociceptive effect of crotalphine in the PGE_2-induced hyperalgesia model in rats. Moreover, this effect was mediated by the release of peripheral dynorphin A, an endogenous agonist of κ-opioid receptors, and this release was dependent on CB_2 receptor activation.

Methods

Peptide synthesis

Crotalphine (<E-F-S-P-E-N-C-Q-G-E-S-Q-P-C, where <E is pyroglutamic acid and 7C-14C forms a disulphide bond; MW 1534.6 Da) was synthesized by the American Peptide Co. (Sunnyvale, CA, USA) as described by Konno *et al.* (2008). The synthesized crotalphine was stored at –20°C until used. The peptide was diluted in sterile saline (0.85% NaCl) and administered either p.o. (gastric cannula, 2 mL) or i.pl., with sterile saline used as a control. The doses of crotalphine were based on previously published results (Konno *et al.*, 2008).

Animals

Male Wistar rats (170–190 g) from the Butantan Institute (São Paulo, Brazil) were used throughout this study. Animals were housed four to five per cage (length, 45 cm; width, 30 cm; height, 20 cm) in a temperature-controlled (21 ± 2°C) and light-controlled (12/12 h light/dark cycle) room. Animals were adapted to these conditions for at least 4 days before the beginning of the experiments. All behavioural tests were performed between 09:00 and 16:00 h. Standard food and water were available *ad libitum* until 2 h prior to p.o. crotalphine administration. After this period, only water was available for a period of no longer than 5 h. All procedures were performed in accordance with the guidelines for the ethical use of conscious animals in pain research published by the International Association for the Study of Pain (Zimmermann, 1983) and were approved by the Institutional Animal Care Committee of the Butantan Institute (CEUAIB, protocol number 622/2009). 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).

Induction of hyperalgesia by PGE_2

Hyperalgesia was induced by i.pl. administration of PGE_2 (100 ng per paw in 0.1 mL of sterile saline) into either one or, when indicated, both hind paws (Picolo *et al.*, 2000; Picolo and Cury, 2004). The nociceptive threshold was measured before and 3 h after injection of the hyperalgesic agent.

Mechanical hyperalgesia evaluation

To assess the nociceptive threshold, the rat paw pressure test was used (Randall and Selitto, 1957) (Ugo Basile, VA, Italy). The test was applied before and 3 h after the i.pl. injection of PGE_2 (100 ng per paw). Testing was blind in regard to group designation. In this test, an increasing force (measured in g) was applied to the hind paw of the rat and interrupted when the animal withdrew its paw. The force necessary to induce this reaction was recorded as the nociceptive threshold. A maximum pressure of 250 g (i.e. cut-off) was established to minimize damage to the paw. To reduce stress, rats were habituated to the testing procedure a day before experimentation.

Experimental design

To evaluate the antinociceptive effect, crotalphine was administered 1 h after PGE_2 (100 ng per paw, 50 μL), by either p.o. (0.008–1.0 $\mu g \cdot kg^{-1}$, 2 mL) or i.pl. (0.0006 μg per paw, 50 μL) routes (Konno *et al.*, 2008). Behavioural tests were conducted 3 h after PGE_2 injection, which corresponds to the peak of the hypernociceptive response induced by PGE_2. To evaluate the involvement of CB_2 receptors in crotalphine-induced antinociception, AM630 (50 μg per paw, 50 μL), a

CB_2 receptor antagonist, was injected i.pl. 30 min before crotalphine administration. To determine the involvement of endogenous opioid peptides in this antinociceptive effect, anti-β-endorphin (0.05–5.0 μg per paw), anti-dynorphin A (0.01–1.0 μg per paw) or anti-met-enkephalin (0.1–50.0 μg per paw) antibodies were given i.pl. 15 min before crotalphine administration. The animals were allocated randomly to each group.

Evaluation of cannabinoid and opioid receptor activation using conformation-sensitive receptor antibodies

To determine which receptor type is activated by crotalphine, conformation state-sensitive anti-CB_1, anti-CB_2, anti-μ, anti-δ and anti-κ antibodies were used. These antibodies are sensitive to activity-mediated conformational changes in the receptors being able to recognize the activated state of the receptor (Gupta *et al*., 2007; Heimann *et al*., 2007; Supporting Information Figs S1–S5). For this study, crotalphine was injected by the i.pl. route (0.0006 μg per paw, right paw) 2 h after PGE_2 administration (100 ng per paw, in 50 μL, right paw) in either the presence or absence of norbinaltorphimine (nor-BNI) and AM630, antagonists of the κ and CB_2 receptors respectively. The antagonists were injected i.pl. 30 min before crotalphine administration. One hour after crotalphine treatment, the rats were anaesthetized with an overdose of ketamine (10%) and xylazine (2%) (1:1 v v^{-1}) i.p., and transcardially perfused with buffered saline followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4) at 4°C. The paw tissue was removed, and the samples were postfixed at 4°C in the perfusion fixative solution for 4 h, cryoprotected in 40% sucrose in PBS for at least 48 h and sectioned at 14 μm on a cryostat in a plane perpendicular to the skin surface and parallel to the long axis of the paw. The sections were mounted on gelatine-treated slides, blocked with 1% BSA + 5% sucrose and processed for immunostaining. The material was incubated overnight with anti-CB_1, anti-CB_2, anti-μ, anti-κ and anti-δ primary antibodies that were selective for the active conformation state of the receptors (Proteimax Biotechnology, Cotia, São Paulo, Brazil), which were labelled with fluorescent Alexa Fluor dyes (682 or 800 nm; Dyomics GmbH, Jena, Germany) and diluted 1:4000. The fluorescence analysis and quantification was performed using the Odyssey system (Li-Cor, Lincoln, NE, USA). The increase in receptor activation was evaluated by accounting for the total number of each receptor expressed in the same tissue using non-conformation-specific antibodies to the κ-opioid receptor-1 (N-19), μ-opioid receptor (N-terminus), δ-opioid receptor (N-terminus), CB_1 receptor (N-terminus) and CB_2 receptor (C-terminus), all of which were diluted 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Measurement of the release of endogenous opioid peptides from rat skin tissue

Measurement of endogenous opioid peptide release was performed as previously described by Ibrahim *et al*. (2005). Crotalphine was dissolved in HBSS (1.26 mM $CaCl_2$, 5.33 mM KCl, 0.44 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.41 mM $MgSO_4$, 138 mM NaCl, 4 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 5.6 mM glucose, pH 7.4) containing 1% BSA. AM630 was dissolved in HBSS/BSA and 0.4% DMSO. Subsequent dilutions were made in HBSS/BSA to obtain the desired final concentration. After the animals were killed using 4% isoflurane inhalation, skin tissue from the plantar surface of the hind paw of both *naïve* rats and rats pretreated with PGE_2 (100 ng per paw) for 2 h was quickly removed using a 4 mm punch and equilibrated for 30 min at 37°C in HBSS/BSA. Each skin sample was transferred to a 1.5 mL polypropylene tube containing 240 μL HBSS/BSA either with or without AM630 (10 μM). Five minutes later, 60 μL of crotalphine solution was added to achieve the desired final concentration. The following groups were incubated at a final volume of 300 μL at 37°C for 30 min with periodic gentle agitation to improve oxygenation: (i) HBSS/BSA alone; (ii) HBSS/BSA containing crotalphine (2.6 nM); (iii) HBSS/BSA containing crotalphine (1 μM); (iv) HBSS/BSA containing crotalphine (2.6 nM) + AM630 (10 μM); (v) HBSS/BSA containing crotalphine (1 μM) + AM630 (10 μM); and (vi) HBSS/BSA containing AM630 (10 μM). The supernatant was collected and kept on ice. β-Endorphin, dynorphin A and met-enkephalin levels in the supernatant were measured immediately using a commercially available enzyme immunoassay (EIA; Bachem, San Carlos, CA, USA).

Materials

AM1241, a CB_2 receptor agonist, was purchased from Cayman Chemical (Ann Arbor, MI, USA). AM630, a CB_2 receptor antagonist, was supplied by Tocris Bioscience (Ellisville, MO, USA). Anti-dynorphin A, anti-β-endorphin and anti-met-enkephalin antisera, as well as the β-endorphin EIA kit, met-enkephalin EIA kit and dynorphin A EIA kit, were obtained from Bachem. PGE_2 was purchased from the Sigma Chemical Co. (St. Louis, MO, USA). Morphine sulphate was kindly provided by União Química (Embu Guaçu, São Paulo, Brazil). AM1241 and AM630 were dissolved in DMSO and then diluted in sterile saline and sterile distilled water, respectively, for injection into the rat paw. The percentage of DMSO in both final solutions was 6%, which was low enough to have no detectable effect in the assay. The anti-dynorphin A, anti-β-endorphin and anti-met-enkephalin antisera were diluted in sterile saline (0.85% NaCl solution). A stock solution of PGE_2 was prepared by dissolving 500 μg PGE_2 in 1 mL of 100% ethanol and then diluting it in sterile saline before injection into the rat paw. The percentage of ethanol in the solution injected in the hind paw was 0.2%.

Nomenclature

The drug and molecular target nomenclature conforms to the *British Journal of Pharmacology's* Concise Guide to PHARMACOLOGY (Alexander *et al*., 2013).

Data analysis

The results are expressed as mean ± SEM. Comparisons between pretreatment and post-treatment as well as between different treatment groups were carried out using the ANOVA (Gad and Weil, 1989) followed by Tukey's *post hoc* test. The results were considered to be statistically significant when $P < 0.05$.

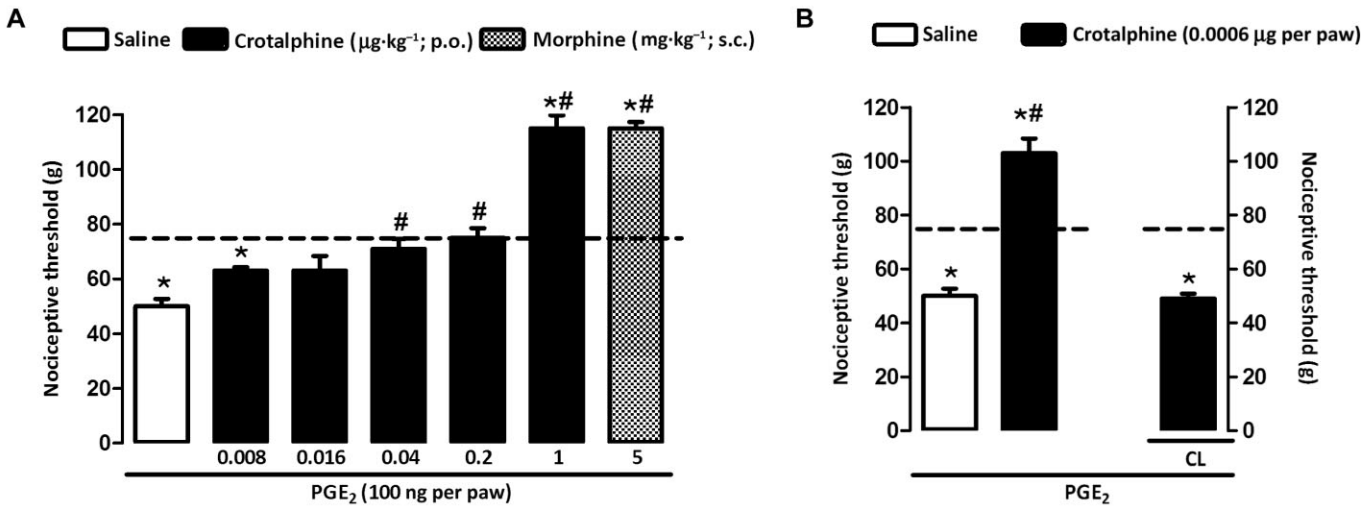

Figure 1

Antinociceptive effect of crotalphine on PGE_2-induced hyperalgesia. Nociceptive threshold was obtained in the rat paw pressure test before (baseline – dotted line) and 3 h after PGE_2 (100 ng per paw) injection. Crotalphine and saline were administered 1 h prior the threshold assessment. (A) Crotalphine (0.008, 0.016, 0.04, 0.2, 1.0 μg·kg^{-1}) administered by p.o. route. Morphine (5 mg·kg^{-1}), administered by s.c. route, was used as a control. (B) Crotalphine (0.0006 μg per paw) administered by i.pl. route. CL, contralateral paw. The results are expressed as means ± SEM (n = 5 animals for control group and 6 animals for experimental group). *P < 0.05 significantly different from values of the baseline measurement (baseline – dotted line); #P < 0.05 significantly different from values of the control group (saline).

Results

Antinociceptive effect of crotalphine on PGE_2-induced hyperalgesia

Our results demonstrated that p.o. (0.008–1.0 μg·kg^{-1}; Figure 1A) and i.pl. (0.0006 μg per paw; Figure 1B) administration of crotalphine caused antinociception in PGE_2-induced mechanical hyperalgesia (Figure 1A and B). The intensity of the increase in the nociceptive threshold of the animals treated with crotalphine was comparable with the increase induced by morphine (5 mg·kg^{-1}, s.c.) (Figure 1A). After injection of PGE_2 into both hind paws and crotalphine (0.0006 μg) into one hind paw, the antihyperalgesic effect of the peptide was detected only in the ipsilateral paw but not in the contralateral one (Figure 1B), indicating that at this dose, the peptide exerts a localized effect.

CB_2 cannabinoid receptors are involved in the antinociceptive effect of crotalphine

The antinociceptive effect of crotalphine administered by either p.o. (1 μg·kg^{-1}; Figure 2A) or i.pl. routes (0.0006 μg; Figure 2B) was blocked by i.pl. injection of AM630, a selective CB_2 cannabinoid receptor antagonist. The peripheral (local) effect of the antagonist was confirmed in experiments in which the CB_2 cannabinoid receptor agonist AM1241 (5 μg per paw), used as positive control, was injected into both hind paws of the rats and the antagonist AM630 into one hind paw. The results demonstrated that AM630 abolishes the antinociceptive effect of the agonist in the ipsilateral paw but not in the contralateral one (Figure 2C).

Crotalphine induces peripheral activation of opioid and cannabinoid receptors

Because our results suggest the involvement of CB_2 receptors in crotalphine-induced antinociceptive effect and previous data from our group showed that peripheral κ-opioid receptors are involved in the effect of the peptide in PGE_2-induced hyperalgesia (Konno *et al.*, 2008), we sought to determine whether crotalphine interferes with the activation state of either cannabinoid or opioid receptors. Paw tissue from animals treated with the peptide (0.0006 μg per paw, i.pl.) and PGE_2 (100 ng per paw) was incubated with anti-CB_1, anti-CB_2, anti-μ, anti-κ and anti-δ receptor antibodies that recognize the activated state of the receptor. These antibodies are able to distinguish conformational changes in the N-terminus of CB_1, CB_2, μ-, κ- and δ-receptors following activation and are thus sensitive to the activity-mediated conformational states of their respective receptors (Gupta *et al.*, 2007; Heimann *et al.*, 2007; Supporting Information Figs S1–S5). The results showed that i.pl. injection of PGE_2 did not alter the activation of CB_1, CB_2, κ- and δ-receptors but decreased the activation of μ-opioid receptors (Figure 3A and B). Intraplantar. injection of crotalphine in the same paw increased the activated state of both CB_2 cannabinoid and κ-opioid receptors (Figure 3A and B). Crotalphine-induced activation of the κ-opioid receptors, but not CB_2 receptors, was blocked by the κ-antagonist nor-BNI. Furthermore, both κ-opioid and CB_2 receptor activation induced by crotalphine was blocked by the CB_2 antagonist AM630 (Figure 3C and D), and crotalphine did not affect the decrease in the activation of μ-opioid receptors induced by PGE_2 (Figure 3B). The fluorescence of the tissue obtained from naïve rats was taken as 100%, and the fluorescence of saline and crotalphine groups,

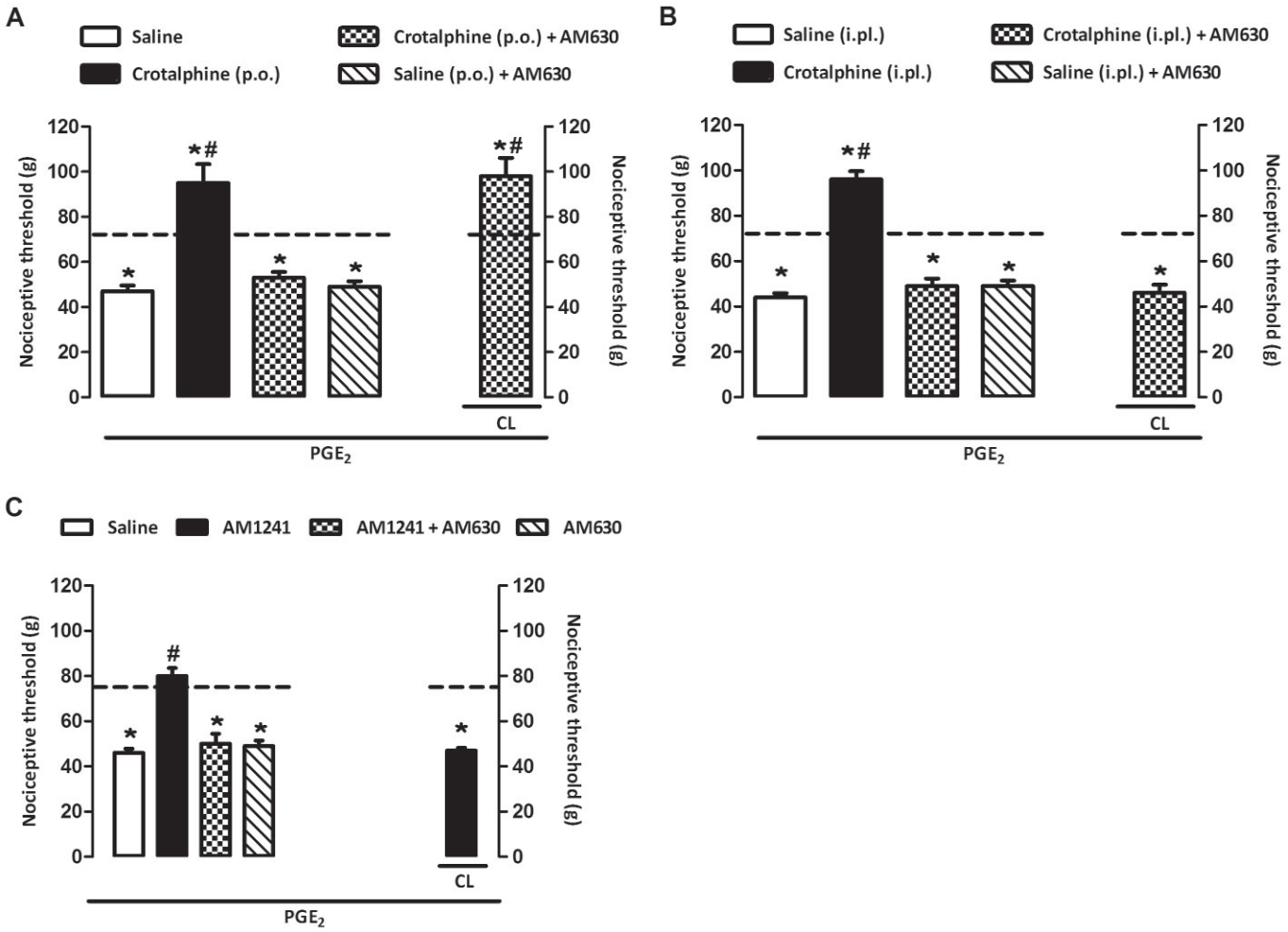

Figure 2

Participation of CB_2 cannabinoid receptors in the antinociceptive effect of crotalphine. Nociceptive threshold was obtained in the rat paw pressure test before (baseline – dotted line) and 3 h after PGE_2 (100 ng per paw) injection. (A) Crotalphine (1 μg·kg^{-1}) was administered by p.o. route 1 h before the threshold assessment. (B) Crotalphine (0.0006 μg per paw) was administered by i.pl. route 1 h before the threshold assessment. (C) AM1241 (10 μg per paw) was administered by i.pl. route 1 h before the threshold assessment. AM630 (CB_2 antagonist, 50 μg per paw, i.pl.) was administered 30 min before crotalphine (A and B) or AM1241 (C). CL, contralateral paw. The results are expressed as means ± SEM (n = 5 animals for control group and 6 animals for experimental group). *P < 0.05 compared with the values of the baseline measurement (dashed line); #P < 0.05 compared with the control group (saline).

acquired using the Odyssey system (Li-Cor), was represented as the percentage of activation in relation to the control group (Figure 3B). The results here presented were normalized to total level of opioid receptors.

Dynorphin A release is responsible for the antinociceptive effect of crotalphine

Our results demonstrated that both CB_2 cannabinoid and κ-opioid receptors are activated after crotalphine injection. Based on data from the literature demonstrating that CB_2 cannabinoid receptor activation produces antinociception by stimulating peripheral endogenous opioid release (Ibrahim *et al.*, 2005), we sought to determine whether endogenous opioids could mediate the antinociceptive effect of crotalphine. Our results suggested that i.pl. administration of the anti-dynorphin A antibody prevented the effect of p.o. administered crotalphine (Figure 4C), whereas anti-β-endorphin and anti-met-enkephalin antibodies did not interfere with this effect (Figure 4A and B). The dose of the anti-dynorphin A antibody induced a localized effect because its injection abolished the antinociceptive effect of the peptide in the injected paw but not in the contralateral one (Figure 4C).

Release of dynorphin A induced by crotalphine depends on CB_2 receptor activation

Because crotalphine induces a peripheral (local) effect, we sought to determine whether the endogenous opioids involved in this effect could be released from the rat paw skin tissue. Tissues obtained from the paws of both naïve animals and rats pretreated with PGE_2 were incubated with

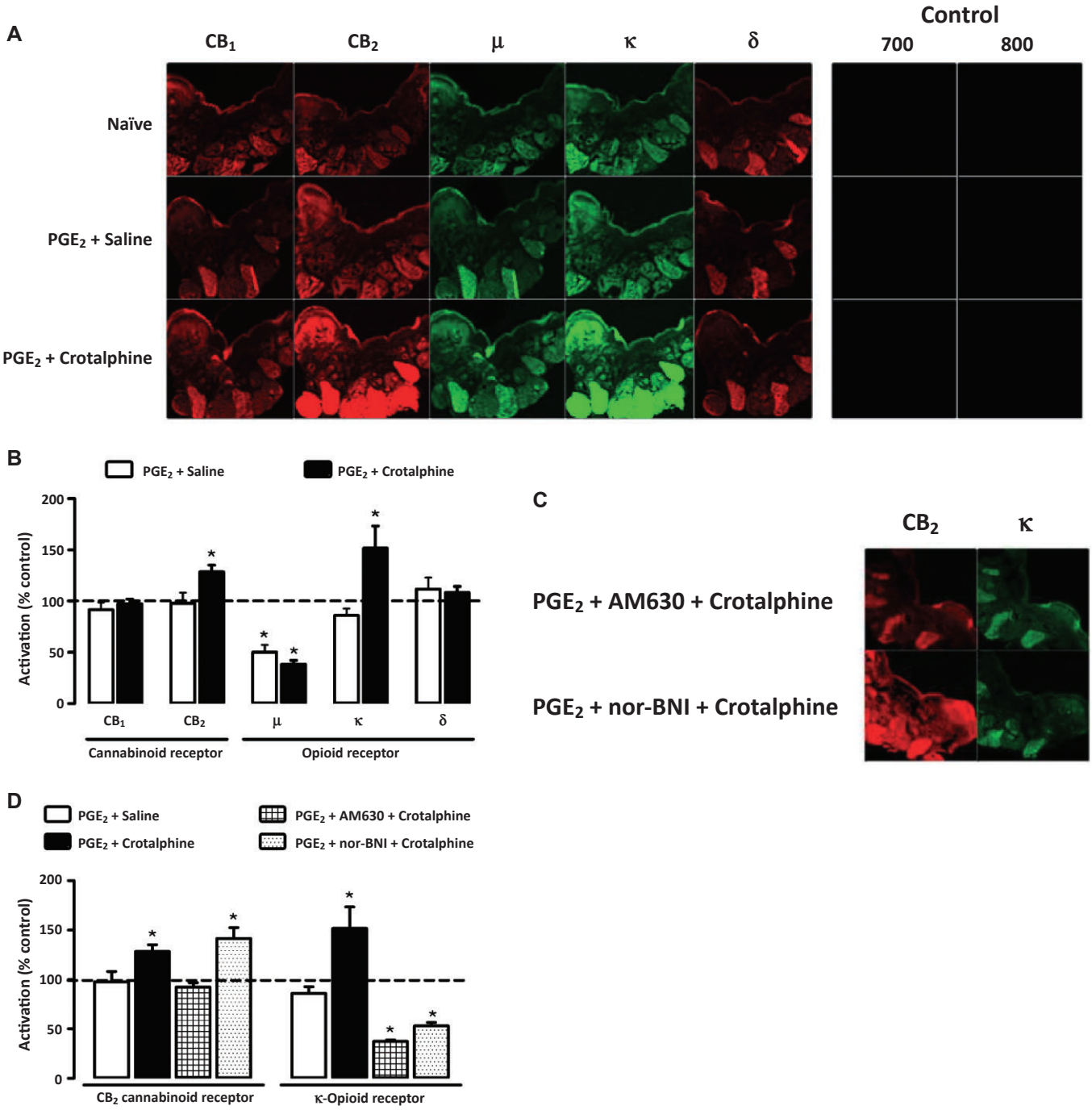

Figure 3

Crotalphine induces an increase in the activation of κ-opioid and CB_2 cannabinoid receptors in skin tissue. PGE_2 (100 ng per paw, i.pl.) and crotalphine (0.0006 μg per paw, i.pl.) were administered in the rat paw 3 and 1 h before the perfusion respectively. (A,B) The immunostaining technique was performed in slices of tissue from the rat paw using conformation state-sensitive anti-CB_1, anti-CB_2, anti-μ, anti-δ and anti-κ antibodies. (C,D) The immunostaining technique was performed in slice of tissue from the rat paw using conformation state-sensitive anti-CB_2 and anti-κ antibodies, in the presence of nor-BNI or AM630, antagonists of κ and CB_2 receptors, respectively, administered by i.pl. route 30 min before crotalphine. (A,C) Images of the sections. Analysis and quantification of fluorescence was performed using the Odyssey system (Li-Cor). (B,D) The fluorescence of the naïve rats was taken as 100% (dashed line), and the fluorescence of the saline and crotalphine groups was represented as percentage of activation compared with the control group. The control group (negative control) represents slices treated only with secondary antibody labelled with fluorescent Alexa Fluor dyes (682 and 800 nm). The results are expressed as means ± SEM (four tissue sections from each rat in each treatment group; $n = 6$ animals for control group and 7 animals for experimental group).*$P < 0.05$ compared with the control group measurement (dashed line).

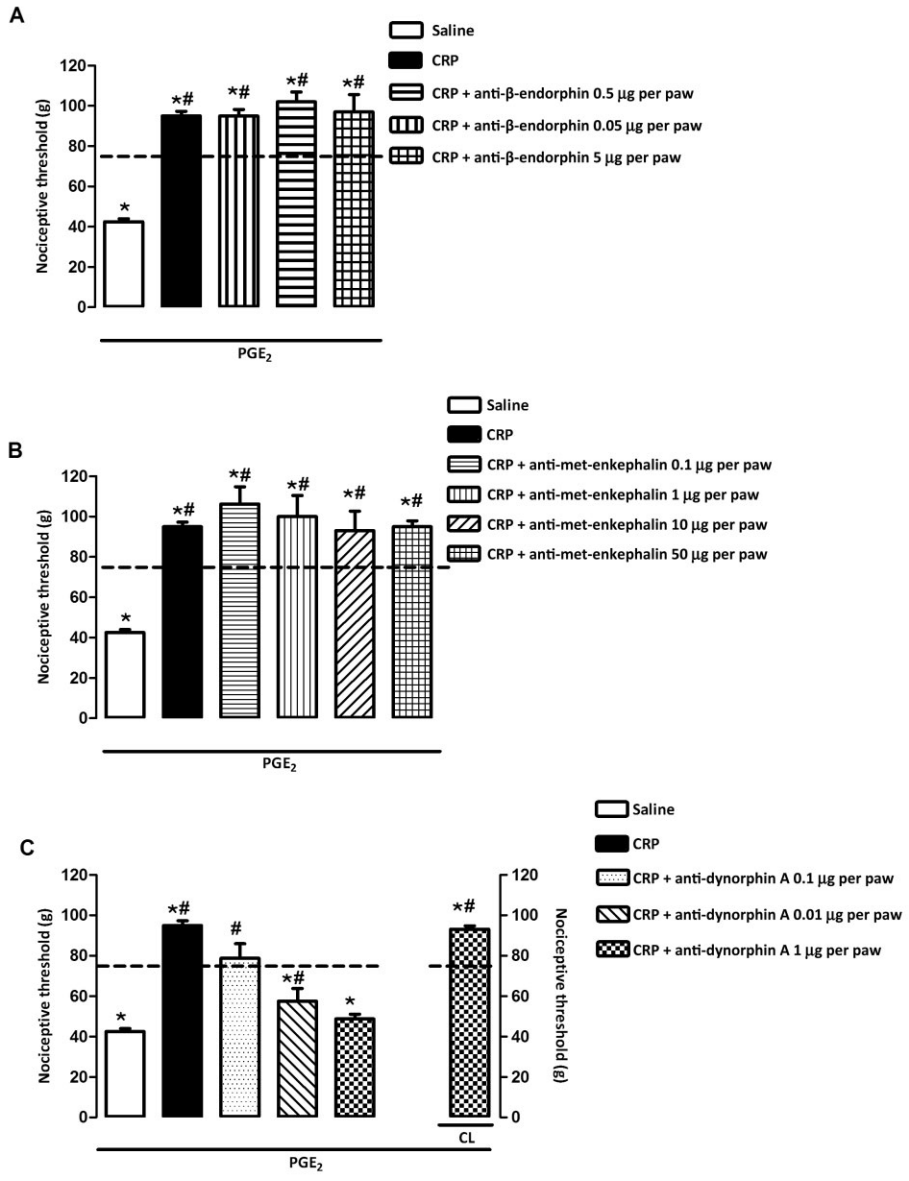

Figure 4

Participation of endogenous opioid in the antinociceptive effect of crotalphine on PGE_2-induced hyperalgesia. Nociceptive threshold was obtained in the rat paw pressure test before (baseline – dotted line) and 3 h after PGE_2 (100 ng per paw) injection. Crotalphine (CRP – 1 µg·kg^{-1}, p.o.) was administered 1 h before the threshold assessment. Antibodies anti-β-endorphin (0.05, 0.5, 5.0 µg per paw, i.pl.; A), anti-met-enkephalin (0.1, 1, 10, 50 µg per paw, i.pl.; B) and anti-dynorphin A (0.01, 0.1, 1.0 µg per paw, i.pl.; C) were administered 15 min before crotalphine. The results are expressed as means ± SEM (n = 5 animals for control group and 6 animals for experimental group).*P < 0.05 compared with the values of the baseline measurement (dashed line); #P < 0.05 compared with the control group (saline).

crotalphine, and the amounts of dynorphin A, β-endorphin and met-enkephalin released into the incubation medium were measured. Incubation of the tissue with two concentrations of crotalphine – 2.6 nM, which corresponds to a dose of 0.0006 µg per paw, and 1 µM, a concentration that activates the intracellular signalling cascade of mediators related to MAPKs *in vitro* (V.O. Zambelli, unpubl. data) – caused the release of dynorphin A from tissues obtained from naïve (Figure 5A) and PGE_2 pretreated (Figure 5B) rats. The amount of dynorphin A released in the presence of PGE_2 (Figure 5B) was significantly higher compared with the amount released by tissues obtained from naïve rats (Figure 5A). This release was prevented by treatment with AM630 (Figure 5).

Discussion

The results presented herein demonstrate for the first time the involvement of the cannabinoid system in the antinociceptive effect of crotalphine. Our results clearly showed that (i) the antinociceptive effect of crotalphine is mediated by dynorphin A, and (ii) the peptide is able to release dynorphin

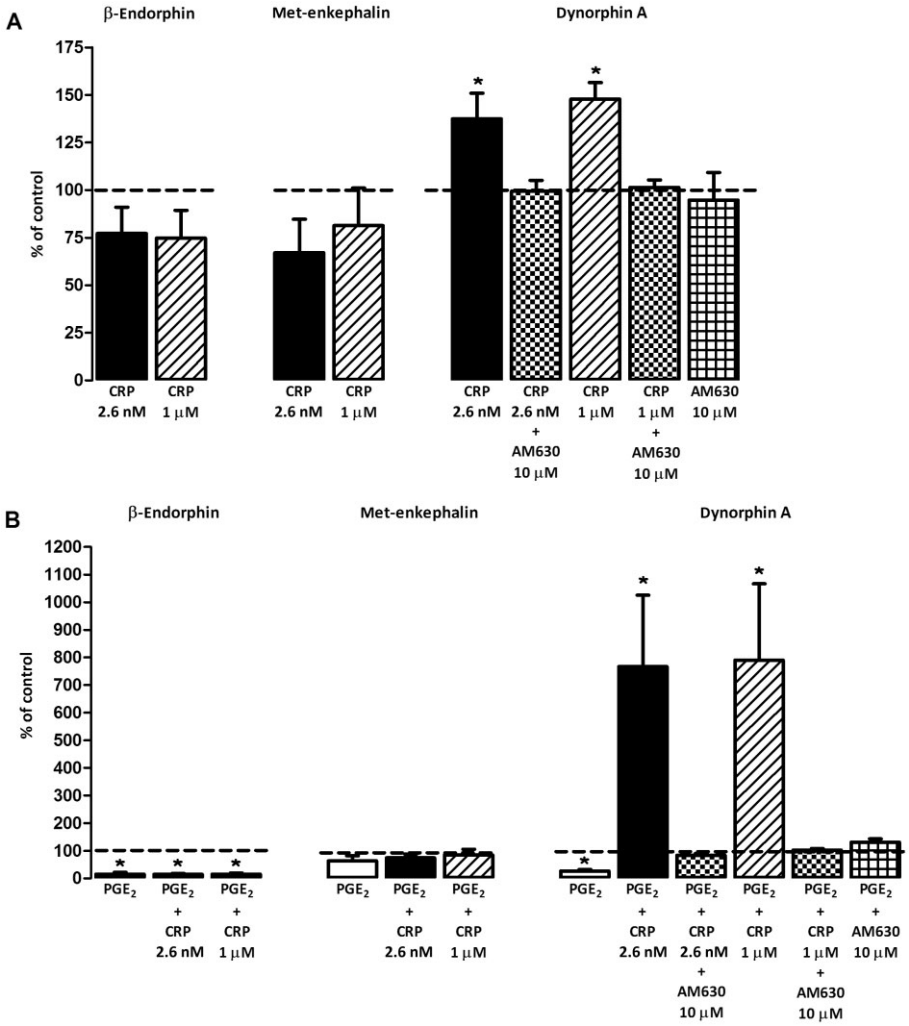

Figure 5

Release of endogenous opioid dynorphin A from rat paw skin tissue induced by crotalphine. For this *in vitro* assay, skin from the plantar surface of the hind paw of *naïve* (A) or PGE_2 pretreated (100 μg per paw, 2 h before; B) rats were collected using a punch (4 mm in diameter to prepare skin samples of equivalent surface area) and placed in HBSS containing crotalphine (2.6 and 1.0 μM) or crotalphine (2.6 and 1.0 μM) plus AM630 (10 μM). β-Endorphin, met-enkephalin and dynorphin A content in the supernatant was measured using a commercially available EIA (Peninsula Laboratories). The endogenous opioid release from the control group (saline rats) was represented as 100% (baseline – dotted line), and the release of crotalphine and crotalphine + AM630 groups was represented as percentage of release in relation to control group. The results are expressed as the means ± SEM ($n = 6$ animals for control group and 7 animals for experimental group). *$P < 0.05$ compared with the control group (dashed line).

A from rat paw skin tissue via a CB_2 receptor-dependent mechanism. Taken together, these results suggest that CB_2 receptor activation induced by crotalphine stimulates the release of endogenous dynorphin A from paw tissue, which then may act on κ-opioid receptors of primary afferent neurons to inhibit nociception.

Cannabinoids exert their analgesic effects acting in the brain via descending modulation, by a direct spinal action and/or by an action on the peripheral nerve (Zogopoulos *et al.*, 2013). The cannabinoid GPCRs CB_1 and CB_2 are the best-studied molecular targets of cannabinoids, which bind to and activate them with different affinity (Basu and Dittel, 2011; Graham *et al.*, 2009). CB_1 is the most abundant GPCR found in the brain, being highly expressed in regions associated with emotion, cognition, memory, motor and executive function (such as cortex, limbic system, hippocampus, cerebellum, brainstem and several nuclei in the basal ganglia) (Ameri, 1999; Piomelli, 2003; Mackie, 2008; Svizenska *et al.*, 2008) and in brain areas involved in nociceptive transmission and processing (including the periaqueductal grey, anterior cingulate cortex and thalamus, in addition to the dorsal horn of the spinal cord and dorsal root ganglion) (Herkenham *et al.*, 1991; Farquhar-Smith *et al.*, 2000; Zogopoulos *et al.*, 2013). CB_1 is also found in peripheral tissues, co-expressed with CB_2, including immune cells, bone cells, adipose tissue, liver, renal tissue and skeletal muscle (Galiègue *et al.*, 1995; Farquhar-Smith *et al.*, 2000; Turu and Hunyady, 2010). CB_2 is predominantly expressed in immune cells throughout the

whole body (i.e. B lymphocytes, macrophages, NK cells), and they are also expressed in the myocardium, the human coronary endothelial cells, the smooth muscle cells, keratinocytes, adipocytes and the liver (Galiègue *et al.*, 1995; Klein *et al.*, 2003; Ashton *et al.*, 2006; Zogopoulos *et al.*, 2013). CB_2 is also present at low levels in some areas of the brain (like perivascular microglial cells, astrocytes, cerebromicrovascular endothelial cells and in brainstem), where it is markedly activated upon insults (Carrier *et al.*, 2004; Gong *et al.*, 2006; Onaivi *et al.*, 2006; Zogopoulos *et al.*, 2013). Considering the predominant expression of CB_2 receptors in the periphery and the previous demonstration of the endogenous opioid release induced by peripheral CB_2 activation (Ibrahim *et al.*, 2005; Su *et al.*, 2011; Katsuyama *et al.*, 2013), we focused this study on the involvement of CB_2 receptors in the antinociceptive activity of crotalphine. Despite the data showing that the analgesic effect of crotalphine is completely reversed by the CB_2 receptor antagonist, we cannot rule out the involvement of CB_1 receptors in this mechanism.

Anyway, the results obtained in the assays using antibodies that are sensitive to active GPCR conformations demonstrated that the CB_2, but not CB_1 receptors, exert increased activity after administration of crotalphine in the PGE_2-induced hyperalgesia. In addition to the activation of CB_2 receptors, these assays also demonstrated an increased activation of κ-opioid receptors induced by crotalphine. These results are in agreement with previous data from our group where it was observed that κ-, but not μ- and δ-, opioid receptors are involved in the crotalphine antinociceptive effect in both PGE_2- and carrageenan-induced hyperalgesia (Konno *et al.*, 2008). It is important to note that PGE_2, *per se*, did not alter the activation of both receptors but inhibited the activity of μ-opioid receptors. Several lines of evidence have indicated that during an inflammatory response, distinct peripheral nociceptive stimuli could activate and/or inhibit the transcription and/or expression of specific opioid receptors in nociceptive fibres (Stein, 1993; Antonijevic *et al.*, 1995; Obara *et al.*, 2009; Stein and Lang, 2009). The decrease in the activation of μ-opioid receptors induced by PGE_2 was not altered by crotalphine.

The investigation of the possible interaction between opioid and cannabinoid systems clearly indicated that crotalphine-induced activation of κ-opioid receptors depends on CB_2 receptor activation, as nor-BNI blocked crotalphine-induced κ-opioid receptor activation but not CB_2 receptor activation, whereas the CB_2 antagonist blocked crotalphine-induced activation of both the κ-opioid receptors and the CB_2 receptors. These results suggest that crotalphine-induced κ-opioid receptor activation is subsequent to the CB_2 cannabinoid receptor activation.

Several biochemical, molecular and pharmacological studies have demonstrated reciprocal interactions between cannabinoid and opioid systems, suggesting a common underlying mechanism (Cichewicz, 2004; Desroches and Beaulieu, 2010). In fact, the endogenous opioid system could be involved in cannabinoid antinociception, and the endogenous cannabinoid system could play a role in opioid antinociception (Smith *et al.*, 1994; Pugh *et al.*, 1996; Ibrahim *et al.*, 2005; da Fonseca Pacheco *et al.*, 2008; Welch, 2009; Desroches and Beaulieu, 2010). As CB_2 and κ-opioid receptors are activated after crotalphine administration and because CB_2 activation may induce the release of endogenous opioids (Ibrahim *et al.*, 2005), we hypothesized that the antinociceptive effect of crotalphine could involve the release of endogenous opioids. This hypothesis was confirmed by the results indicating that dynorphin A is involved in the antinociceptive effect of crotalphine in the PGE_2-induced hyperalgesia. We also demonstrated through *ex vivo* experiments that the action of crotalphine involves the release of dynorphin A from rat paw tissue, which is blocked by a CB_2 receptor antagonist. These data may explain the involvement of the κ-opioid receptors in crotalphine-induced antinociceptive effect because dynorphin A is an endogenous agonist of these opioid receptors (Chavkin *et al.*, 1982).

The release of endogenous opioids by cannabinoid receptor activation has been shown previously by Ibrahim *et al.* (2005). These authors demonstrated that CB_2 receptor activation produces antinociception by stimulating β-endorphin release from keratinocytes, which, in turn, produces antinociception by acting on μ-opioid receptors. Our data suggest, for the first time, that activation of the CB_2 receptors, which are present in skin tissue, may induce the local release of dynorphin A, and this release may occur in non-injured tissue. The results showing that the amount of β-endorphin and met-enkephalin released from the paw tissue was not altered by crotalphine support our previous data demonstrating that μ- and δ-opioid receptors are not involved in the antinociceptive action of crotalphine in PGE_2-induced hyperalgesia (Konno *et al.*, 2008).

The cell type involved in crotalphine-induced dynorphin A release has not been characterized. Both resident and migrated cells may release endogenous opioids after different types of stimuli (Cabot *et al.*, 1997; 2001; Cabot, 2001; Ibrahim *et al.*, 2005; Sehgal *et al.*, 2011). Aside from keratinocytes (Ibrahim *et al.*, 2005), other components of skin such as resident immune and inflammatory cells may release opioid peptides. Immune cells, including T and B lymphocytes, granulocytes, monocytes and macrophages, are the major source of endogenous opioid ligands in the skin due to their abundance compared with resident keratinocytes and peripheral sensory neurons (Stein *et al.*, 1990; Hassan *et al.*, 1992; Schauer *et al.*, 1994; Hadley and Haskell-Luevano, 1999; Cabot, 2001; Cabot *et al.*, 2001). Further investigation is needed to determine the cells responsible for the release of dynorphin A by crotalphine.

In conclusion, our results demonstrated that the antinociceptive effect of crotalphine involves activation of peripheral CB_2 receptors, which stimulates dynorphin A release from peripheral skin tissues. Dynorphin A, in turn, induces antinociception by acting on κ-opioid receptors expressed on primary afferent neurons. These results contribute to the understanding of the mechanisms involved in crotalphine-induced antinociception and corroborate the existence of a close connection between the opioid and cannabinoid systems in the control of pain.

Acknowledgements

The authors are grateful to Dr Lakshmi A. Devi (Mount Sinai School of Medicine, New York) for critical suggestions on these studies. We also express our gratitude to Dr Luiz Roberto

G. de Britto for allowing us to perform the rat paw slices in his laboratory, and to Adilson da S. Alves and Miriam Aline Geigner for their technical assistance. This work was supported by funds from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Brazil, Grant Nos. 2009/14203-5 and 2010/12917-8), Instituto Nacional de Ciencia e Tecnologia em Toxinologia (INCTTOX PROGRAM) of Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)/FAPESP (Grant No. 2008/57898-0), and Fundação do Desenvolvimento Administrativo (FUNDAP).

Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.12488

Figure S1 SKNSH cells (human neuroblastoma) treated with (±)-U-50488 hydrochloride (a κ agonist). SKNSH cells (10^6 cells per well) endogenously expressing κ-receptors were treated with the indicated concentrations of (±)-U-50488 hydrochloride. Cells were incubated overnight at 4°C with 1 μg per well of antireceptor antibodies, then washed three times with PBS and incubated with a secondary antibody (dilution 1:500) of alkaline phosphatase-conjugated anti-rabbit antibodies for 60 min at room temperature. After washing three times with PBS, the amount of binding was detected using phosphatase substrate. The extent of receptor recognition by the antibodies was assayed by ELISA. Data from the vehicle-treated cells were taken as 100%. All of the results of the experiments are represented as the percentage of the control. The results are expressed as mean ± SEM (n = 3).

Figure S2 Rat spleen membranes treated with Δ^9-THC. Rat spleen membranes (5 μg per well) were treated with 1 μM concentration of either Δ^9-THC (CB_2 agonist) or WIN 55212-3 (CB_2 antagonist). Cells were incubated overnight at 4°C with 1 μg per well of antireceptor antibodies, washed three times with PBS and incubated with a secondary antibody (dilution 1:500) of alkaline phosphatase-conjugated anti-rabbit antibodies for 60 min at room temperature. After washing three times with PBS, the amount of binding was detected using phosphatase substrate. The extent of receptor recognition by the antibodies was assayed by ELISA. Data from the vehicle-treated cells were taken as 100%. All of the results of the experiments are represented as the percentage of the control. The results are expressed as means ± SEM (n = 3).

Figure S3 *In vivo* assay for the κ-opioid receptor. Mice were injected with either saline or 10 $mg \cdot kg^{-1}$ of dynorphin A (i.p.) and killed 30 min after the injection. Brains were immediately collected and stored at –80°C until used. The immunostaining technique was performed in tissue slices from the mouse brain using conformation state-sensitive anti-κ antibodies. Primary antibodies were labelled with fluorescent dyes, and fluorescence was measured using Odyssey equipment from Li-Cor. (A) Images from brain sections

representing striatum from the control and treated mice were probed with conformation-specific anti-κ-opioid receptor antibodies. (B) Data quantification from striatum-treated and control mice. Vehicle-treated mice were taken as 100%. The results are expressed as means ± SEM (n = 3).

Figure S4 HEK293 cells expressing the CB_2 receptor treated with Δ^9-THC. HEK293 cells expressing the CB_2 receptor were treated with 1 μM concentration of agonist (Δ^9 -THC). The cells were incubated overnight at 4°C with 1 μg per well of anti-receptor antibodies, washed three times with PBS and incubated with a secondary antibody (dilution 1:500) of alkaline phosphatase-conjugated anti-rabbit antibodies for 60 min at room temperature. After washing three times with PBS, the amount of binding was detected using phosphatase substrate. The extent of receptor recognition by the antibodies was assayed by ELISA. Data from the vehicle-treated cells were taken as 100%. All of the results of the experiments are represented as a percentage of the control. The results are expressed as means ± SEM (n = 3).

Figure S5 Specificity of the conformation-sensitive receptor antibodies. Paw tissues from animals treated with either ACEA (CB_1 agonist) or AM1241 (CB_2 agonist) 2 h after PGE_2 (100 ng per paw) injection were incubated with anti-CB_1 and anti-CB_2 conformation-sensitive receptor antibodies (Figure 5A). Paw tissues from animals treated with DAMGO (μ-agonist), U50.488 (κ-agonist) or DPDPE (δ agonist) 2 h after PGE_2 (100 ng per paw) injection were incubated with anti-μ, anti-κ and anti-δ conformation-sensitive receptor antibodies (Figure 5B). The analysis and quantification of the fluorescence was performed using the Odyssey system (Li-Cor). The fluorescence of the naïve rats was taken as 100% (dashed line), and the fluorescence of the treated groups was represented as percentage of activation compared with the control group. This control group (negative control) represents slices treated only with secondary antibody labelled with fluorescent Alexa Fluor dyes (682 and 800 nm). The results are expressed as means ± SEM (n = 5 animals for control group and 6 animals for experimental group). *P < 0.05 compared with the control group measurement (dashed line).

Table S1 Cross-reactivity assay. SKNSH cells (human neuroblastoma) were treated with various agonists (1 μM). Cells were incubated overnight at 4°C with 1 μg per well of anti-receptor antibodies, washed three times with PBS and incubated with a secondary antibody (dilution 1:500) of alkaline phosphatase-conjugated anti-rabbit antibodies for 60 min at room temperature. After being washed three times with PBS, the amount of binding by the cells was detected by a phosphatase substrate. The extent of receptor recognition by the antibodies was assayed by ELISA. Data from the vehicle-treated cells were taken as 100%. The results are represented as the percentage of the control (buffer treatment). Data from the vehicle-treated cells were taken as 100%. The results are expressed as means ± SD (n = 3).