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Increases in vanilloid TRPV1 receptor protein and CGRP content during endotoxemia in rats ☆

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Abstract

The aim of the present study was to determine whether the transient receptor potential vanilloid (TRPV1) receptor protein as well as the calcitonin gene-related peptide (CGRP) content could be enhanced after the i.p. administration of 5 mg/kg lipopolysaccharide (LPS) to Sprague–Dawley rats. In tongue tissue, used as a representative model of TRPV1 receptors expression, there was a significant increase in the abundance of TRPV1 receptor protein 6 h after LPS administration. In mesenteric arteries, the density of the CGRP-positive nerves as well as the release of CGRP induced by 10 μM anandamide was also significantly increased 6 h after LPS administration. The relaxant responses induced by anandamide in mesenteric beds isolated from either untreated or LPS-treated rats were abolished after a 2 h exposure to 10 μM capsaicin. Moreover, anandamide-induced relaxations of untreated mesenteries were potentiated by the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA, 0.1 μM), but not by its inactive analogue 4α-phorbol (0.1 μM). The potentiation of anandamide effects caused by the PKC activator was accompanied by a significant increase in the overflow of CGRP induced by anandamide in the untreated rats. It is proposed that the overexpression of the TRPV1 receptors and the increased content of CGRP could contribute to the enhancement of anandamide effects during the endotoxemic shock. An eventual phosphorylation event linked to the overflow of CGRP could also participate in the enhanced relaxation caused by anandamide in endotoxemia.

Keywords: Anandamide; TRPV1; CGRP; PKC; Vasorelaxation; Septic shock

1. Introduction

The induction of proinflammatory gene expression by bacterial lipopolysaccharide (LPS) plays a crucial role in triggering the cardiovascular collapse experienced by patients with systemic Gram negative bacterial infection, a major cause of morbidity and mortality (Karima et al., 1999). Increased circulating levels of anandamide, substance that has been proposed as a mediator in endotoxin-induced hypotension, were found under septic conditions (Varga et al., 1998; Kohro et al., 2004).

Enhancement of anandamide effects has been observed in several pathological situations, such as hypertensive states (Mendizabal et al., 2001; Li et al., 2003), cholestasis (Moezi et al., 2004) and cirrhosis (Domenicali et al., 2005). Potentiation of relaxant effects of anandamide in the mesenteric vasculature has also been reported for an early stage of septic shock, *i.e.* 6 h after intraperitoneal administration of 5 mg/kg of lipopolysaccharide (Orliac et al., 2003). Anandamide-induced relaxations were reported to be antagonized by the TRPV1 receptor antagonist capsazepine in mesenteric arteries isolated from either untreated (Mendizabal et al., 2001) or LPS-treated rats (Orliac et al., 2003). Relaxations to capsaicin were also enhanced in mesenteric beds of LPS-treated rats (Orliac et al., 2003).

The activation of transient receptor potential vanilloid 1 (TRPV1) receptors by anandamide has been proposed to have

☆ This work is dedicated to the beloved memory of Maria Luz Orliac, who suddenly passed away on March 21, 2006.

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potential implications on inflammatory and cardiovascular disorders (Ross, 2003). Since vasorelaxant responses to anandamide in the rat vascular mesenteric bed are mainly mediated through the activation of TRPV1 receptors in perivascular sensory nerves and coupled to the release of calcitonine gene-related peptide (CGRP) (Zygmunt et al., 1999), the aim of the present work was to study whether TRPV1 receptor protein as well as CGRP content could be enhanced during septic shock. In addition, based on the evidence that sensitization of TRPV1 receptors is linked to an increased phosphorylation that results from the enhancement of protein kinase C (PKC) activity (Premkumar and Ahern, 2000), we also studied whether the direct activation of PKC through phorbol esters could potentiate the vasorelaxant effects of anandamide in the untreated rats.

2. Materials and methods

2.1. Animal treatment

Male Sprague–Dawley rats weighting between 230–350 g were housed under 12:12-h light:dark cycle, at controlled room temperature with food and water *ad libitum*. Experiments were conducted in accordance with the Helsinki Declaration on research involving animals and human beings. Six hours prior to the beginning of the functional and biochemical studies, a single injection of LPS from Escherichia coli (5 mg/kg, i.p.) was administered in 0.25 ml saline/ 100 g body weight. The untreated rats received the same volume of saline. During this 6-h period, food and water were available *ad libitum*. The intraperitoneal injection of LPS could be considered a good model to evaluate the early stages of sepsis because it avoids the rapid increase in CGRP plasmatic levels (Tang et al., 1997) and its consequent severe hypotension (Huttemeier et al., 1993) achieved when the endotoxin is directly drawn to blood stream by intravenous injection.

2.2. Vascular reactivity

Six hours after the i.p. administration of 5 mg/kg LPS, rats were anaesthetized with 1.2 g/kg urethane, the abdomen was opened and the mesenteric bed was cannulated and removed according to the method described by McGregor (1965). The isolated mesenteric bed was transferred to a perpex chamber at 37 °C and perfused with Krebs solution bubbled with 95% O_2/ 5% CO_2 (in mM: NaCl 118; KCl 4.7; $MgCl_2$ 1.2; NaH_2PO_4 1.0; $CaCl_2$ 2.6; $NaHCO_3$ 25.0; glucose 11.1; final pH 7.4) at a constant flow of 2 ml/min maintained by a peristaltic pump. The rate of perfusion was selected on the basis of previous studies that showed that this experimental approach allowed reproducible anandamide-induced relaxations on the consecutive contractions elicited by bolus injections of noradrenaline (Mendizabal et al., 2001). Changes in vascular resistance were measured as changes in perfusion pressure and recorded by a Statham transducer connected to a Grass polygraph. The mesenteric bed was allowed a settling period of 60 min after mounting, before starting the experiments. The basal perfusion pressure of the mesenteric beds was, throughout the entire study, between 20 and 25 mm Hg for both untreated and LPS-treated rats.

After an equilibration period of 60 min at 37 °C the mesenteric beds isolated from LPS-treated as well as from age-matched untreated rats were constricted with bolus injections of noradrenaline. The dose of noradrenaline was selected as to produce a pressure response of 40 to 70 mm Hg, usually attained with 3 to 10 nmol noradrenaline. When an entire concentration-response curve to noradrenaline was performed no differences in the contractile responses were observed between LPS-treated and age-matched untreated rats (Orliac et al., 2003). Since short-lasting contractile responses were highly reproducible throughout the experiment, consecutive injections of noradrenaline were performed up to nine times in each preparation, 20 min apart, as previously reported (Mendizábal et al., 2000).

Concentration-response curves to either anandamide (0.01–10 μM) or capsaicin (0.01–100 nM) were performed by evaluation of the reductions on the contractile responses to noradrenaline. Increasing concentrations of the agonists were perfused 20 min before and during the bolus injections of noradrenaline. Results were expressed as the percent reduction of the pressor effect of noradrenaline before drug perfusion.

In order to study the effects of protein kinase C (PKC) activation on the TRPV1-mediated relaxant responses, either the PKC activator phorbol 12-myristate 13-acetate (0.1 μM PMA) or its inactive analogue 4α-phorbol (0.1 μM) were perfused 40 min before and simultaneously with increasing concentrations of either anandamide or capsaicin. Neither PMA, nor 4α-phorbol had any effect *per se* on the contractile responses elicited by noradrenaline during the time course of the experiments.

A 2 hour perfusion with 10 μM capsaicin followed by a 1 hour period of washout was applied to cause either desensitization and/or CGRP depletion in perivascular sensory nerves, according to Zygmunt et al. (1999). Contractile responses to noradrenaline were unmodified by this treatment. The basal tone was unmodified by any of the concentrations employed of the drugs under study.

Concentrations of capsaicin and PMA were selected on the basis of reported evidence (Orliac et al., 2003; Premkumar and Ahern, 2000). PMA was not tested in LPS-treated rats because under this condition it caused *per se* the reduction of the noradrenaline-induced contractions.

2.3. Western blotting for TRPV1 receptor

2.3.1. Mesenteric bed homogenate

For each individual experiment samples of a pool of three mesenteric beds cleaned to remove connective tissue were homogenized under liquid nitrogen at 4 °C. Homogenization buffer, approximately 8 ml containing 20 mM Tris, 2 mM EGTA, 2 mM EDTA, protease inhibitor mixture (1 tablet/2 g of tissue; Complete®, Roche Diagnostics) and 1 mM PMSF, final pH 7.4, was added until obtain a limpid supernatant after centrifugation at 1100 ×*g* for 10 min. The pellet was resuspended in loading buffer (62.5 mM Tris pH 6.80, 1% w/v SDS, 10% v/v glycerol, 3.5 N 2-mercaptoethanol).

2.3.2. Tongue homogenate

For each individual experiment samples of a pool of three tongues were homogenized at 4 °C in homogenization buffer. The homogenates were centrifuged at 1100 ×*g* for 15 min and the supernatant was centrifuged at 10,000 ×*g* for 10 min. The corresponding supernatant was then centrifuged at 100,000 ×*g* for 1 h. The 100,000 ×*g* pellet was resuspended in the homogenization buffer and loading buffer was added.

In both tissues, the proteins were separated in a sodium dodecyl sulfate-gel containing 8% w/v acrylamide/bisacrilamyde, transferred onto a polyvinylidene fluoride membrane and stained with ponceau red to evaluate the adequate protein transference. Membranes were blocked for 30 min with 5% w/v non-fat milk TTBS (50 mM Tris, 150 mM NaCl, 0.2% Tween 20, final pH 7.50) and incubated overnight with an anti-C-terminal TRPV1 receptor antibody (Sigma Aldrich, St. Louis, MI) 1/700 diluted in 0.5% w/v non-fat milk TTBS. After that, membranes were incubated with anti-rabbit-horseradish peroxidase conjugated antibody (1/4000 diluted in TTBS) for 2 h at room temperature and washed 1 h with TTBS. The bands were detected by autoradiography using enhanced chemiluminescence (ECL Amersham Biosciences) and quantified by densitometric analysis using ImageJ software (1.34S National Institutes of Health USA).

2.4. Immunohistochemistry for CGRP

Animals (230–350 g) were deeply anesthetized with urethane (1.2 g/kg body weight) and subjected to transcardiac perfusion initially with 150 ml of saline solution containing 50 IU of heparin and subsequently with 400 ml of a fixative solution containing 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Mesenteric vascular beds were quickly dissected, cleaned to remove connective tissue and kept in 70% ethanol. Blockade of endogenous peroxidase activity was achieved by incubation at room temperature in 1% H_2O_2 in PBS (0.15 M NaCl, 0.01 M Na_2HPO_4, pH 7.4) for 60 min. Subsequently, the preparations were washed with 70% ethanol and then treated whit 0.2% Triton X-100 (in PBS), before overnight incubation at room temperature with 1/3000 anti-CGRP antibody (Sigma Aldrich, St. Louis, MI) diluted in PBS. The tissues were rewashed with 0.2% Triton X-100 for 1 h, incubated with 1/200 goat anti-rabbit peroxidase conjugated antibody (Sigma Aldrich, St. Louis, MI) diluted in PBS and washed once more with 0.2% Triton X-100. Development of peroxidase activity was performed in 0.1 M acetate buffer, pH 6, containing 1.2 mmol/l diaminobenzidine plus 2.5% (w/v) nickel ammonium sulphate and 0.3% (v/v) H_2O_2 for 15 min at room temperature. To stop the enzymatic reaction, tissue sections were washed in 0.1 M acetate buffer, pH 6. Preparations were mounted on gelatine-coated slides, dehydrated with xylene and coverslipped using Permount for light microscopic observation. All groups were simultaneously processed to prevent interassays differences. Specificity of the inmunohistochemical procedure was assessed by omitting the primary antibody.

2.4.1. Morphometric measurement

All measurements were performed by different observers to ensure objectivity. CGRP-immunoreactive fibres were explored in myenteric plexus preparations. Only tissue sections corresponding to second arterial branches were included in the quantification, ensuring that chosen regions were equivalent among experiments. Mesenteries were separated in two parts for the CGRP-incubated as well as for the corresponding control. The antibody labelled a varicose network of CGRP-immunoreactive fibres that was not observed in the control tissues. In each mesenteric bed, immunoreactive structures were measured in six to eight fields corresponding to different branches to avoid overlapping of the measured fields using an *Eclipse* 50*i* NIKON light microscope equipped with a video camera Nikon DS-SM. Relative area (stained/total area) per field was measured to evaluate CGRP positive structures. Specific immunoreactivity was the difference between anti-CGRP-incubated tissues and the corresponding controls. The analog images were digitalized into an array of 2560 × 1920 pixels corresponding to an area of 312 μm × 234 μm (400× magnification). To ensure specific immunoreactivity analysis, a threshold setting, that was kept constant along each experiment, was determined by using a range of grey value selected as to allow the segmentation between the specific signals (*i.e.* CGRP-containing fibres) from the background. Morphometric analysis was performed with Image J software (1.34S National Institutes of Health USA).

2.5. Radioimmunoassay for CGRP

Mesenteric bed perfusates (2 ml/min) obtained in either basal conditions (Krebs solution) or in the presence of 10 μM anandamide were collected in consecutive 5 min samples, 60 min after an equilibration period at 37 °C. The samples were collected in polypropylene tubes, acidified with acetic acid (final concentration 0.1 N) and maintained on ice. Each sample was applied to an activated Oasis® HLB solid-phase extraction cartridge (Waters Corporation, Milford, MA) and the adsorbed peptide was eluted with 2 ml of 60% acetonitrile in 0.1% trifluoroacetic acid. The eluate was vacuum-dried and stored at −70 °C until radioimmunoassay (RIA). The samples were preincubated with 1/5000 rabbit anti-rat CGRP serum (Sigma Aldrich, St. Louis, MI) diluted in RIA buffer (0.1 M phosphate buffer containing 50 mM NaCl, 0.1% bovine albumin, 0.1% Triton X-100 and 0.01% NaN_3) at 4 °C overnight. The reaction mixture was incubated with rat ([^{125}I]–Tyr^0)-alpha-CGRP (Peninsula Laboratories, Inc., San Carlos, CA) (10,000 cpm/tube, diluted in RIA buffer) for 2 h at 4 °C. The antibody-bound antigen was separated from free antigens by incubation with 1/5 anti-rabbit IgG serum (Sigma Aldrich, St. Louis, MI) plus 5% rabbit serum for 90 min. After that, 6% polyethyleneglycol 8000 was added and the mixture was centrifuged at 3000 rpm during 20 min. The radioactivity in the pellets was measured in a solid scintillation gamma counter. The lower detection limit was 2 pg/tube for CGRP.

2.6. Drugs

Lipopolysaccharides from *Escherichia coli* Serotype 055:B5, (−)-noradrenaline bitartrate, phorbol 12-myristate 13-acetate (PMA), 4α-phorbol, capsaicin and anti-actin antibody were obtained from Sigma-Aldrich, St. Louis, MO. Anandamide was

purchased from Cayman Chemical Company, Ann Arbor, MI. Anandamide, capsaicin, PMA and 4α-phorbol were dissolved in ethanol. The remaining drugs were dissolved in distilled water. The maximal concentration of ethanol employed (0.1%) had no effect *per se* on the contractile responses elicited by noradrenaline in the mesenteric beds.

2.7. Statistical analysis

Data were presented as the mean±S.E.M. Statistical comparisons were made by either two ways analysis of variance followed by Bonferroni's *post hoc t*-test or one way analysis of variance followed by Dunnett's multiple comparison test or Student's *t*-test for paired data. A *P* value smaller than 0.05 was considered significant.

3. Results

3.1. Changes in TRPV1 receptor protein levels

To test the possibility that changes in TRPV1 receptor protein expression could occur during endotoxemia, Western blot analysis of the TRPV1 receptor protein were performed in mesenteric bed as well as in tongue homogenates isolated from either untreated or LPS-treated rats. Fig. 1A shows a Western blot of mesenteric tissue where two bands of 120 kDa and 100 kDa of molecular weight were detected with the anti-TRPV1 antibody. These bands were obtained in a unique experiment carried out after several unsuccessful attempts. On the other hand, tongue was an excellent tissue to work with and it was previously employed as a model of TRPV1 receptor expression (Ishida et al., 2002; Kido et al., 2003). Fig. 1B shows that the representative Western blot image of the TRPV1 receptor protein obtained in tongue homogenates presented a unique band of 100 kDa. In both tissues a higher intensity of the bands was observed in LPS-treated *versus* untreated rats under equal protein loading conditions, assessed with either ponceau red stain or anti-actin antibody in vascular and tongue homogenates, respectively. Relative densitometric analysis performed in tongue tissue showed a significant increase in the expression of the TRPV1 receptor protein after 6 h of LPS treatment (Fig. 1, lower panel).

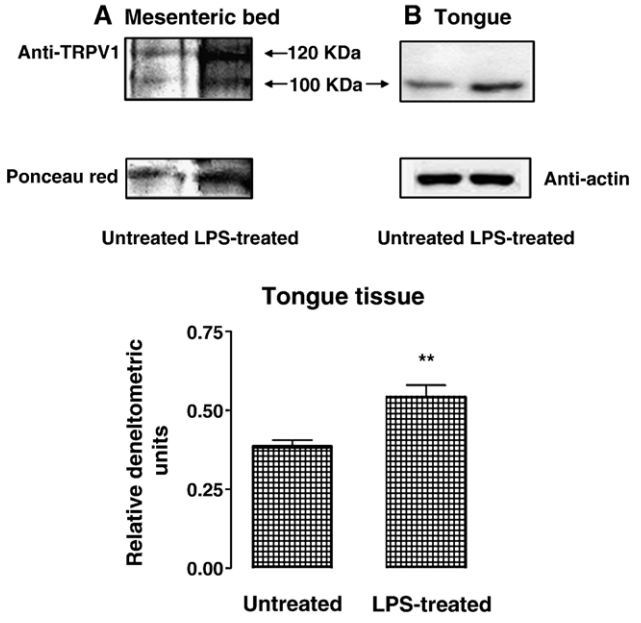

Fig. 1. Effects of LPS treatment on the expression of TRPV1 receptors. Upper panel: TRPV1 receptor expression in mesenteric bed homogenates (in A) and in tongue homogenates (in B) isolated from untreated and LPS-treated rats (5 mg/kg i.p., for 6 h). Homogenates represent a pool of three tissues. Actin (50 kDa) in tongue or a protein stained with ponceau red (37 kDa) in mesenteries were employed as internal standards to control sample loading. Lower panel: Relative densitometric analysis in tongue tissue ($n=4$) performed by the computer Image J program. $^{**}P<0.01$ when compared to the corresponding controls.

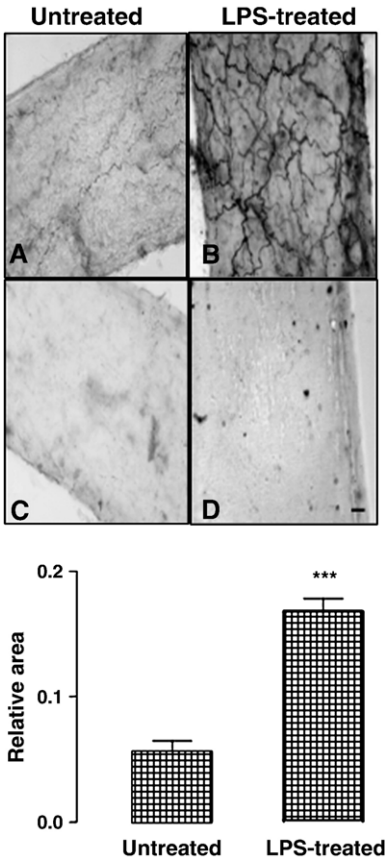

Fig. 2. Effects of LPS treatment on CGRP-immunoreactivity in the rat vascular mesenteric bed. Upper panel: Representative sections of myenteric CGRP-immunostained perivascular nerve terminals from untreated (left) and LPS-treated rats (5 mg/kg i.p., for 6 h, right). The tissues were incubated with either 1/3000 anti-CGRP antibody (A and B) or buffer solution (C and D). The antibody labelled a varicose network of CGRP-immunoreactive fibres that was not observed in non-antibody-incubated tissues. The photomicrographs were captured at 400× magnification; the scale bar in D indicates 50 μm. Lower panel: Bars represent the mean± S.E.M. ($n=4$) of relative morphometric units of a relative area (stained/total area) per field. Specific immunoreactivity was calculated as the difference between anti-CGRP-incubated tissues and the corresponding non-primary antibody-incubated controls. $^{***}P<0.0001$ compared to the corresponding controls.

3.2. Changes in CGRP content

Immunohistochemical analysis showed the typical distribution of CGRP nerve fibres surrounding second and third order

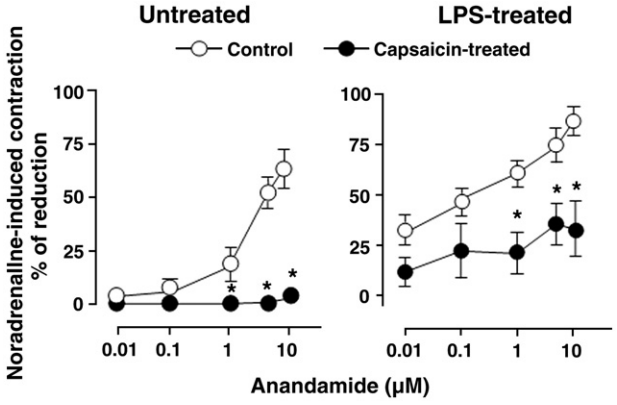

Fig. 3. Effects of capsaicin treatment (10 μM capsaicin for 2 h) on the anandamide-induced reductions of the contractile responses to noradrenaline in mesenteric beds isolated from untreated and LPS-treated rats (5 mg/kg i.p., for 6 h). Increasing concentrations of anandamide were perfused 20 min before and during a bolus injection of a dose of noradrenaline (3–10 nmol) that caused an increase in the perfusion pressure of 40 to 70 mm Hg in mesenteric beds of either untreated or LPS-treated mesenteries. Results are expressed as the percent reductions caused by anandamide on the initial contractile response to noradrenaline. Control mesenteries are depicted in open circles and capsaicin-treated mesenteries in filled circles. Results are the mean±S.E.M. of 5–6 experiments per group. *$P<0.05$ compared to the corresponding control values.

mesenteric arteries. As shown in Fig. 2, CGRP-positive immunoreactivity was significantly increased in mesenteric beds isolated 6 h after LPS treatment when compared to untreated tissues. Further evidence of the participation of CGRP in the relaxant responses to anandamide was obtained by the observation (Fig. 3) that the relaxant responses induced by anandamide were almost entirely abolished, both in the untreated and in the LPS-treated rats, after sensory *in vitro* denervation through a 2-h exposure to 10 μM capsaicin. Moreover, the contractile responses to noradrenaline observed in the presence of the two higher concentrations of anandamide in denervated mesenteries isolated from septic rats (35±8 mm

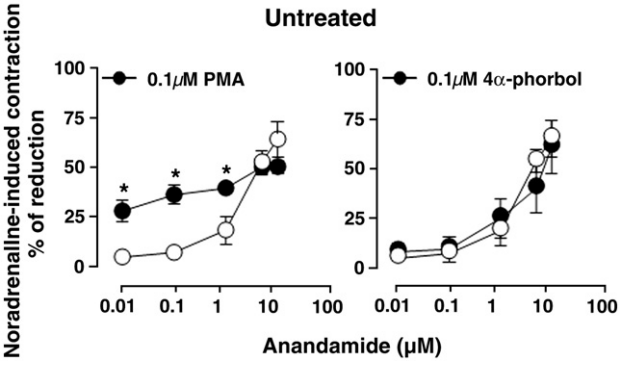

Fig. 4. Effects of PKC activation on the anandamide-induced reductions of the contractile responses to noradrenaline in mesenteric beds isolated from untreated rats. The isolated mesenteric beds were perfused with either the PKC activator 0.1 μM PMA or its inactive analogue 0.1 μM 4α-phorbol. Drugs were perfused 40 min before and simultaneously with the increasing concentrations of anandamide. The initial contraction to noradrenaline was induced 40 min after either PMA or 4α-phorbol perfusion. Results are the mean±S.E.M. in the controls (open circles, $n=4$) and drug-treated groups (filled circles, $n=6$). *$P<0.05$ compared to the corresponding control values.

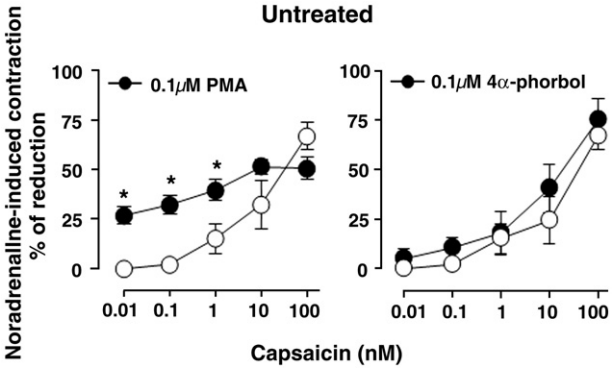

Fig. 5. Effects of PKC activation on the capsaicin-induced reductions of the contractile responses to noradrenaline in mesenteric beds isolated from untreated rats. The isolated mesenteric beds were perfused with either the PKC activator 0.1 μM PMA or its inactive analogue 0.1 μM 4α-phorbol. Drugs were perfused 40 min before and simultaneously with the increasing concentrations of capsaicin. The initial contraction to noradrenaline was induced 40 min after either PMA or 4α-phorbol perfusion. Results are the mean±S.E.M. in the controls (open circles, $n=5$) and drug-treated groups (filled circles, $n=6$). *$P<0.05$ compared to the corresponding control values.

Hg and 37±12 mm Hg for anandamide 5 and 10 μM, respectively) did not differ from the contraction induced by noradrenaline under control conditions (50±6 mm Hg) thus precluding the possibility of a residual relaxation that it seemed to be apparent from the curves depicted in Fig. 3.

3.3. Participation of PKC in TRPV1 receptor sensitization

To study the possibility that direct PKC activation could increase TRPV1-mediated relaxant responses, the effects of the PKC activator PMA were tested on the relaxations caused by either anandamide (Fig. 4) or the vanilloid receptor agonist capsaicin (Fig. 5). The perfusion with 0.1 μM PMA but not with its inactive analogue 0.1 μM 4α-phorbol significantly potentiated submaximal vasorelaxant responses to either anandamide (Fig. 4) or capsaicin (Fig. 5) in mesenteric beds isolated from untreated rats.

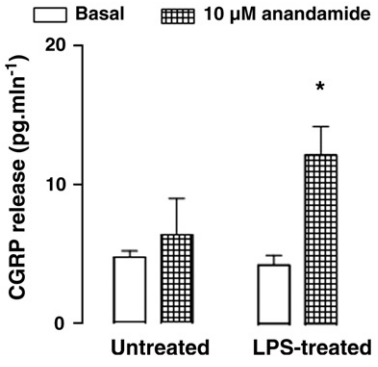

Fig. 6. Effects of LPS treatment on the anandamide-induced release of CGRP in rat vascular mesenteric bed perfusates. CGRP release under basal conditions (open bars) and after incubation for 10 min with 10 μM anandamide (hatched bars) in either untreated or LPS-treated rats (5 mg/kg i.p., for 6 h), as indicated at the bottom of the figure. Results are the mean±S.E.M. of 3–4 experiments per group. *$P<0.05$ when compared to the corresponding basal values.

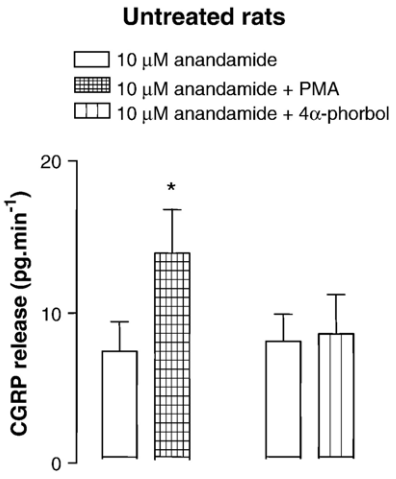

Fig. 7. Effects of PKC activation on the anandamide-induced release of CGRP in rat vascular mesenteric bed perfusates from untreated rats. The CGRP release in the presence of 10 μM anandamide (open bars) was also measured after coincubation with either 0.1 μM PMA (cross hatched bar) or 0.1 μM 4α-phorbol (hatched bar). Results are the mean±S.E.M. of 3–4 experiments per group. *$P<0.05$ when compared to the corresponding control values.

3.4. Changes in CGRP release

To evaluate whether the enhancement of anandamide-induced relaxations caused by LPS-treatment could be linked to a greater release of CGRP, the overflow of CGRP from mesenteric bed perfusates was assayed in untreated as well as in LPS-treated rats. As shown in Fig. 6, the basal CGRP release did not differ between untreated and LPS-treated preparations. Nevertheless, the release of CGRP was significantly increased in LPS-treated but not in untreated rats when the mesenteric beds were perfused during 10 min with 10 μM anandamide (Fig. 6). On the other hand, the release of CGRP induced by 10 μM anandamide in untreated rats (Fig. 7) was significantly enhanced by the PKC activator 0.1 μM PMA but not by its inactive analogue 4α-phorbol. Basal CGRP values were not modified by either drug tested (data not shown).

4. Discussion

The vanilloid TRPV1 receptor is a member of a superfamily of transient receptor potential ion channels that are gated by capsaicin, extracellular acidic pH and noxious heat (Caterina et al., 1997; Tominaga et al., 1998; Dedov and Roufogalis, 2000). The present observation that 6 h after i.p. administration of 5 mg/kg LPS to rats the abundance of vanilloid TRPV1 receptor protein as well as the CGRP content are increased, gives further support to the proposal that vanilloid TRPV1 receptors can contribute in triggering deleterious effects, such as those found in sepsis (Varga et al., 1998; Kohro et al., 2004), haemorrhagic shock (Randall et al., 2002) and cirrhosis (Domenicali et al., 2005).

To study the TRPV1 receptor expression, Western blots of mesenteric bed tissue were performed. After several unsuccessful attempts, we get a Western blot that showed higher densities at 120 kDa (97%) as well as at 100 kDa (20%) in LPS-treated compared to untreated rats. Due to the high amount of protein that had to be loaded in order to detect TRPV1 receptor in vascular tissue, it was not possible to use actin as an internal control of protein loading, hence a band of approximately 37 kDa observed under ponceau red stain was used for the control of loading in mesenteric arteries. Interestingly, a band of 120 kDa that matches with the band of the same molecular weight observed in vascular tissue in the present experiments has also been reported in ganglia and in primary culture of rat trigeminal neurons (Simonetti et al., 2006). The 120 kDa band deserves further studies for the characterization of the TRPV1 receptor in vascular tissues.

As the manipulation of vascular tissue was very tedious and difficult, we continued the study with samples of tongue, that it had previously been employed as a systemic model for TRPV1 receptor expression (Ishida et al., 2002; Kido et al., 2003). In this latter preparation, a band of approximately 100 kDa found in untreated as well as in LPS-treated rats is coincident with the molecular weight described for TRPV1 receptors in several rat tissues, namely nociceptive fibres of either sensory dorsal root or trigeminal ganglia (Caterina et al., 1997), stomach (Nagahama et al., 2003) and brain (Toth et al., 2005).

The present observation that the abundance of vanilloid receptor protein is enhanced under endotoxemia, agrees with the increases in TRPV1 receptor protein reported for other injuries such as acid reflux by pyloric ligation (Nagahama et al., 2003), noxious heat (Vass et al., 2004) and streptozotocin-induced diabetes (Hong and Wiley, 2005). In addition, potentiation of gating of the TRPV1 receptors by capsaicin, protons, heat and anandamide was also found in primary sensory neurons from neonatal rats, dorsal root ganglia and human embryonic kidney (HEK) cell line (Vellani et al., 2001).

The fact that 6 h after LPS administration the increase in CGRP content in perivascular sensory nerves was not accompanied by an enhancement in the basal levels of this peptide in mesenteric bed perfusates could suggest that endogenous factors linked to septic shock such as protons, heat and anandamide are not altered at early endotoxemia. On the other hand, the observation that CGRP release was indeed increased when exogenous anandamide was added to the LPS-treated mesenteries agrees with the observed increment in the abundance of vanilloid TRPV1 receptor protein and could indicate a TRPV1 receptor sensitization to anandamide effects at early stages of endotoxemia. Although to substantiate these findings it would be interesting to know whether relaxation to anandamide is blocked by a CGRP antagonist, our experimental model of perfused arteries makes hardly unaffordable the employment of a CGRP antagonist. On the other hand, prevention of CGRP release after capsaicin treatment is also worth to be done and it is in fact the aim of forthcoming experiments.

Pretreatment of mesenteric beds with capsaicin, substance that activates primary sensory nerves that then become refractory to the subsequent stimuli because of desensitization and/or CGRP depletion (Szallasi and Blumberg, 1999), showed that the relaxant effects of anandamide were almost abolished

and suggested that the participation of CGRP is likely to be mandatory for anandamide relaxations. Nevertheless, and in spite of this functional evidence, biochemical studies such as changes in TRPV1 expression as well as changes in CGRP content after capsaicin pretreatment are worth to be done in the future and would provide further confirmation of the role of CGRP on endocannabinoids vascular effects.

In addition, the possibility exists that in LPS treated rats, the reductions of anandamide responses after capsaicin pretreatment had resulted from interference of the upregulation of TRPV1 receptors through protein kinase C (PKC)-induced phosphorylation. In support of this view is the observation that activation of PKC reverses capsaicin-induced desensitization of TRPV1 ion channels in dorsal root ganglion neurons and in TRPV1 transfected Chinese hamster ovarian cells (Mandadi et al., 2004). Nevertheless, this possibility is precluded in the present study since the relaxations to anandamide were indeed significantly reduced after capsaicin pretreatment in mesenteries isolated from LPS rats, where PKC was proposed to sensitize TRPV1 vanilloid receptors (Premkumar and Ahern, 2000).

Since CGRP is a potent hypotensive agent (Wang et al., 1992; Arnalich et al., 1996), the systemic overexpression of vanilloid TRPV1 receptors as well as the increased vascular content of CGRP and the enhanced sensitivity to anandamide after LPS treatment could account, among several other factors, for the development of the hypotension that characterizes septic shock. Factors that may be important include upregulation of inducible nitric oxide synthase expression and increased NO release (Han et al., 2004), increased release of prostanoids (Dugo et al., 2004), decreased secretion of vasopressin (Treschan and Peters, 2006), and change in the expression of catecholamine receptors (Matsuda et al., 2000).

An alternative hypothesis has been proposed by Okajima et al. (2005) who suggested that CGRP may contribute to the reduction of endotoxin-induced hypotension through an increase of the endothelial production of prostanoids. Nevertheless, the observation in the Okajima' study that hypotension is indeed induced in endotoxemic rats at the time when the release of CGRP was increased, is not likely to support a protective role for CGRP in sepsis.

The potentiation of the relaxant responses to anandamide as well as the increase in the anandamide-induced CGRP release found after the *in vitro* exposure to the PKC activator PMA could suggest that phosphorylation contributes to the potentiation of anandamide responses observed in sepsis. Furthermore, the fact that basal CGRP release was not modified by PMA *per se* could indicate that activation of vanilloid TRPV1 receptors requires, in addition to phosphorylation, the eventual increase in endogenous factors linked to the septic noxa.

Direct phosphorylation of TRPV1 receptors through PKCε has been reported for the potentiation caused by ATP in the TRPV1 receptor currents evoked by capsaicin and protons in HEK cells (Numazaki et al., 2002). Moreover, time-dependent increases in isotype-specific PKC (α and ε isotypes) activity have been reported in rat aorta with a maximal expression after 3 h *in vitro* treatment with 30 ng/ml LPS (McKenna et al., 1997). In addition to the participation of PKC in TRPV1 receptor activity, other protein kinases such as PKA have also shown to induce CGRP release in dorsal root ganglia neurons of neonatal rats (Hou and Wang, 2001).

It is concluded that enhancement of anandamide effects during the endotoxemic shock is likely to involve the overexpression of vanilloid TRPV1 receptors and the increased content of CGRP. The phosphorylation of the vanilloid TRPV1 receptors could contribute to the enhanced relaxation caused by anandamide in endotoxemia. Nevertheless, further studies to unmask direct phosphorylation of vanilloid TRPV1 receptors in mesenteric arteries, as those carried out by Numazaki et al. (2002) in HEK cells, should be performed to elucidate the latter point and it is in fact the aim of our forthcoming experiments.

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