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Enhancement of the hypotensive effects of intrathecally injected endocannabinoids by the entourage compound palmitoylethanolamide ☆

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ABSTRACT

The intrathecal (i.t.) injection of 50 and 100 nmol anandamide to urethane anesthetized rats induced a dose-dependent decrease in the mean blood pressure (-10.6 ± 1.6 mmHg and -15.0 ± 1.7 mmHg, respectively; $n = 6$) whereas a lower dose of this endocannabinoid (25 nmol) was devoid of effect. Similar responses were obtained both with the non-metabolizable analog methanandamide and with the endocannabinoid *N*-arachidonoyldopamine. When the sub-effective dose (25 nmol) of each compound was co-injected with palmitoylethanolamide (100 nmol), significant decreases in the blood pressure were observed ($-12.3 + 1.3$ mmHg for anandamide; -12.1 ± 0.8 mmHg for methanandamide; -12.1 ± 0.8 mmHg for *N*-arachidonoyldopamine; $n = 4$–6). Palmitoylethanolamide also enhanced the hypotensive responses to the 50 nmol-dose of both anandamide and methanandamide. The hypotensive response induced by co-administration of palmitoylethanolamide and 25 nmol anandamide was prevented both by the cannabinoid CB_1 receptor antagonist SR 144716A (20 nmol; i.t.) and by the vanilloid TRPV1 receptor antagonist capsazepine (20 nmol; i.t.) and enhanced by pretreatment with URB602 (3.5 nmol; i.t.), a putative inhibitor of palmitoylethanolamide degradation. These results suggest that in the spinal cord palmitoylethanolamide acts as an entourage compound for the hypotensive effects of i.t. administered endocannabinoids. The facilitative action of palmitoylethanolamide affects the vanilloid TRPV1 as well as the cannabinoid CB_1 receptor-mediated effects of endocannabinoids on the blood pressure control.

1. Introduction

Some biological effects of endocannabinoids are enhanced by related endogenous fatty acid derivatives that do not produce those effects *per se*. This facilitative action termed entourage effect was observed for the first time by Ben-Shabat et al. (1998) who characterized the potentiation of a tetrad of cannabinoid receptor-mediated responses to 2-arachidonoylglycerol by other endogenous monoacylglycerols in mice. Entourage properties have also been reported for oleamide (Hiley and Hoi, 2007; Lambert and Di Marzo, 1999), some *N*-acyl dopamines (De Petrocellis et al., 2004) and several saturated *N*-acyl ethanolamines such as palmitoylethanolamide (De Petrocellis et al., 2001, 2002; Lambert and Di Marzo, 1999; Smart et al., 2002) which are co-synthesized and co-released with endocannabinoids. The entourage effect of palmitoylethanolamide has been reported to be caused either to a facilitative action at vanilloid TRPV1 receptors (De Petrocellis et al., 2001, 2002) or to the protection of endocannabinoids such as anandamide from enzymatic degradation by the fatty acid amide hydrolase (Di Marzo et al., 2001; Smart et al., 2002).

Palmitoylethanolamide and anandamide are present in the spinal cord (Baker et al., 2001; Maccarrone et al., 2001). Since the intrathecal (i.t.) injection of either anandamide or the metabolically stable analog methanandamide to urethane anesthetized rats produces hypotensive effects related to the activation of both cannabinoid CB_1 and vanilloid TRPV1 spinal receptors (García et al., 2003), the aim of the present study was to examine whether palmitoylethanolamide could be an entourage compound for the hypotensive responses caused by i.t. administration of endocannabinoids.

2. Methods

2.1. Surgical procedures

This study was performed in accordance with the guide for the Care and Use of Laboratory Animals of the National Research Council (USA, 1996). Procedures for the evaluation of the cardiovascular effects after intrathecal (i.t.) injection of drugs were similar to those previously described (García et al., 2003, 2006). Male Sprague–Dawley rats (250–350 g) were maintained in a room with controlled room temperature (19–22 °C) and 12 h light–dark cycle. Food and water were freely available. The animals were anesthetized with urethane (1.2 g/kg, intraperitoneal route). A polyethylene cannula was placed in the right femoral artery for recording of the blood pressure. For i.t. injection of drugs, a cannula (outside diameter: 0.65 mm) was inserted into the

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subarachnoid space at the level of the T_1–T_2 vertebrae and gently pushed downward as described by Dib (1984). The tip of the cannula was positioned at the level of the T_{12}–L_1 intervertebral space. The position of the cannula was verified post-mortem by direct observation after opening the ventral aspect of the vertebrae. Animals in which the cannula tip was not at the level of the T_{12}–L_1 intervertebral space ±2.0 mm were not used for subsequent data analysis. The body temperature was kept at 37–38 °C by a heating lamp.

2.2. Blood pressure recording and heart rate calculation

The blood pressure was measured at the right femoral artery via a Statham P23 1D transducer and recorded on a Grass 7B polygraph (Quincy, MA, USA). Baseline values were recorded for at least 30 min before starting the experiment. The mean blood pressure was calculated from the formula: diastolic pressure +1/3 (systolic pressure–diastolic pressure). The heart rate was calculated from the blood pressure record. Changes in mean blood pressure and heart rate induced by i.t. injection of drugs refer to the differences between the values obtained just before the beginning of the drug injection and the values at a given time.

2.3. Experimental protocols

Intrathecal injections were administered through a Hamilton syringe and consisted of 10 μl of either palmitoylethanolamide (100 nmol) or palmitoylethanolamide vehicle followed by 10 μl of anandamida, methanandamide, *N*-arachidonoyldopamine (25, 50 and 100 nmol) or the corresponding vehicles. Additional saline (7 μl) was injected to clear the drug solutions from the catheter. The solutions (total volume 27 μl) were loaded in the syringe immediately before injection and were administered within 1 min. The blood pressure was recorded just before (time 0) and at the following times after the beginning of the i.t. injection of drugs: 1, 2, 4, 6, 8, 10, 15, 20, 25 and 30 min. When indicated, the animals were pretreated with one of the following drugs: the vanilloid TRPV1 receptor antagonist capsazepine (20 nmol; i.t.), the cannabinoid CB_1 receptor antagonist SR 141716A (20 nmol; i.t.) or the monoacylglycerol lipase inhibitor URB602 (3.5 nmol; i.t.). Doses of drugs were selected on the basis of the studies by Suplita et al., 2006 (URB602) and García et al., 2003, 2006 (other compounds).

To examine whether i.t. injected endocannabinoids diffuse along the bulbospinal axis from the site of injection, some animals received 10 μl palmitoylethanolamide vehicle plus [arachidonoyl-^{3}H]-anandamide (100 nmol in 10 μl; specific activity 0.19 mCi/mmol) plus 10 μl saline, and were sacrificed by decapitation 30 min after the injection. The spinal cord was exposed and cut into 1 cm segments both in the rostral and in the caudal direction starting at the place where the catheter tip was localized. The medulla oblongata was also isolated. The tissue samples were weighed, homogenized in 0.5 N NaOH and kept overnight at room temperature. After that, the tissue homogenates were incubated for 3 h at 50 °C and centrifuged at 3000 g. The radioactivity per gram of wet tissue was measured in the supernatants by liquid scintillation spectrometry and was expressed as a percentage of the total radioactivity present in whole spinal cord and medulla oblongata.

2.4. Drugs

Anandamide, methanandamide and (1, 1'-biphenyl)-3-yl-carbamic acid cyclohexyl ester (URB602) were obtained from Cayman Chemical Co. (USA). Palmitoylethanolamide and *N*-arachidonoyldopamine were obtained from Sigma Chemical Co. (USA). Capsazepine was purchased from Tocris Cookson (USA). N-Piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide (SR 141716A) was a gift from Sanofi Recherche (France). [Arachidonoyl 5, 6, 8, 9, 11, 12, 14, 15,-^{3}H]-anandamide (specific activity 200 Ci/mmol) was purchased from New England Nuclear (USA).

Anandamide, methanandamide and *N*-arachidonoyldopamine were supplied as ethanol solutions and diluted with saline. Palmitoylethanolamide was dissolved in ethanol and further diluted with 10% (w/v) hydroxypropyl-β-cyclodextrin. Capsazepine and UBR602 were dissolved in ethanol and further diluted with saline. Final solutions contained not more than 14% ethanol (v/v). SR 141716A was dissolved in 10% (w/v) hydroxypropyl-β-cyclodextrin.

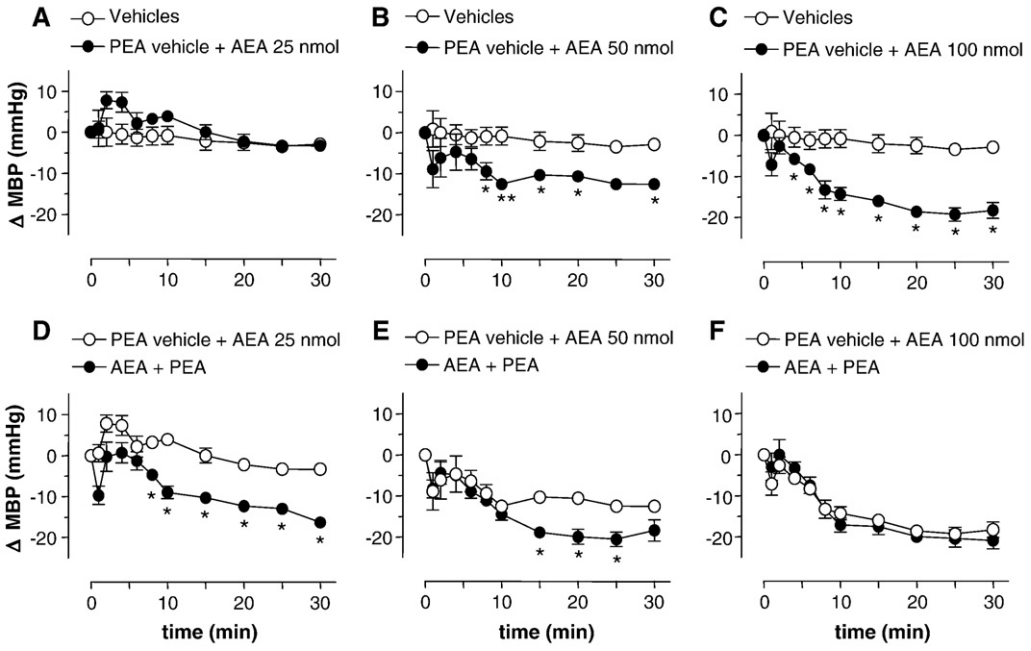

Fig. 1. Effect of palmitoylethanolamide (PEA; 100 nmol; i.t.) on the mean blood pressure changes (ΔMBP; mmHg) induced by i.t. injection of anandamide (AEA). A, B and C: AEA (filled symbols) or AEA vehicle (10 μl 14% ethanol in saline; open symbols) was co-administered with PEA vehicle (10 μl 14% ethanol in 10% hydroxypropyl-β-cyclodextrin) at time 0. D, F and G: AEA was co-administered with either PEA (filled symbols) or PEA vehicle (open symbols). Shown are mean values ± S.E.M. of 4–6 animals per group. *$p<0.01$ vs. the corresponding control value.

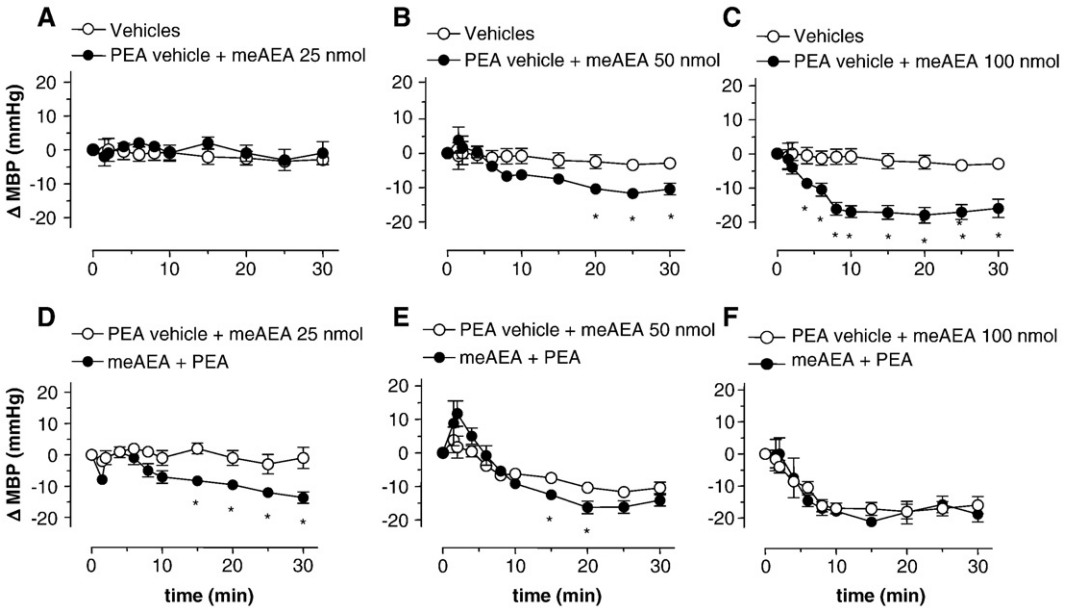

Fig. 2. Effect of palmitoylethanolamide (PEA; 100 nmol; i.t.) on the mean blood pressure changes (ΔMBP; mmHg) induced by i.t. injection of methanandamide (meAEA). A, B and C: meAEA (filled symbols) or meAEA vehicle (10 μl 14% ethanol in saline; open symbols) was co-administered with PEA vehicle (10 μl 14% ethanol in 10% hydroxypropyl-β-cyclodextrin) at time 0. D, E and F: meAEA was co-administered with either PEA (filled symbols) or PEA vehicle (open symbols). Shown are mean values ± S.E.M. of 4 animals per group. *$p<0.01$ vs. the corresponding control value.

2.5. Statistics

All values represent the mean ± S.E.M. Statistical differences were assessed by Student's *t*-test. *P* values smaller than 0.05 were regarded as significant.

3. Results

The resting mean blood pressure and heart rate values in urethane anesthetized rats under control conditions were 67.1 ± 1.6 mmHg and 385 ± 4, respectively ($n = 10$).

The i.t. injection of anandamide induced a dose-dependent decrease in the mean blood pressure, i.e. significant decreases of about 10 and 20 mmHg were obtained with 50 and 100 nmol anandamide, respectively (Fig. 1B and C), whereas 25 nmol was devoid of effect (Fig. 1A). Nevertheless, when 25 nmol anandamide was co-injected with 100 nmol palmitoylethanolamide a significant hypotensive effect was produced (Fig. 1D). The co-administration of palmitoylethanolamide also enhanced the hypotensive response to 50 nmol anandamide (Fig. 1E) but did not modify the blood pressure response induced by 100 nmol anandamide (Fig. 1F). No changes in the mean blood pressure were produced *per se* by 100 nmol palmitoylethanolamide (−2.4 ± 1.1 mmHg; $n = 4$).

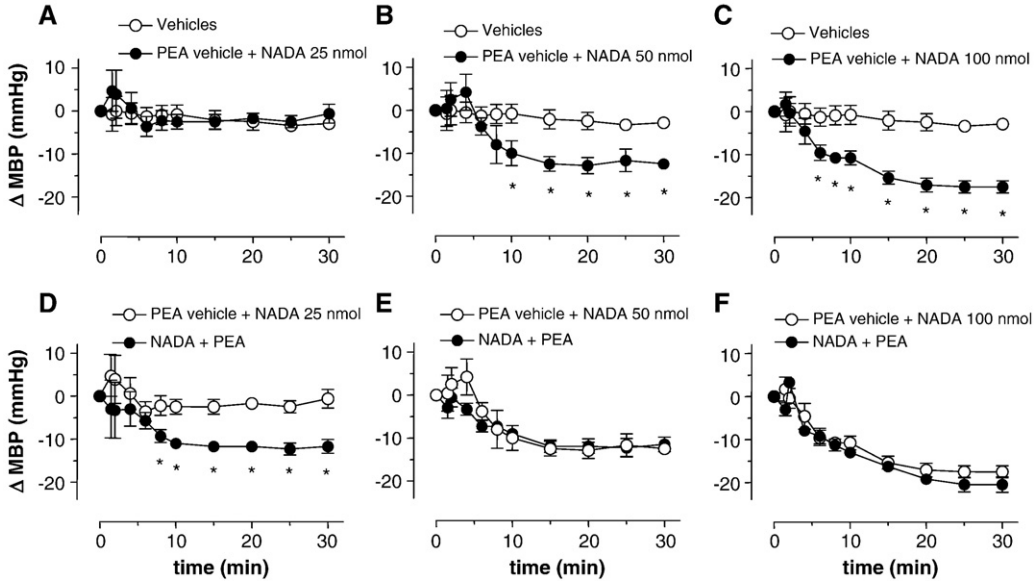

Fig. 3. Effect of palmitoylethanolamide (PEA; 100 nmol; i.t.) on the mean blood pressure changes (ΔMBP; mmHg) induced by i.t. injection of *N*-arachidonoyldopamine (NADA). A, B and C: NADA (filled symbols) or NADA vehicle (10 μl 14% ethanol in saline; open symbols) was co-administered with PEA vehicle (10 μl 14% ethanol in 10% hydroxypropyl-β-cyclodextrin) at time 0. D, E and F: NADA was co-administered with either PEA (filled symbols) or PEA vehicle (open symbols). Shown are mean values ± S.E.M. of 5 animals per group. *$p<0.05$ vs. the corresponding control value.

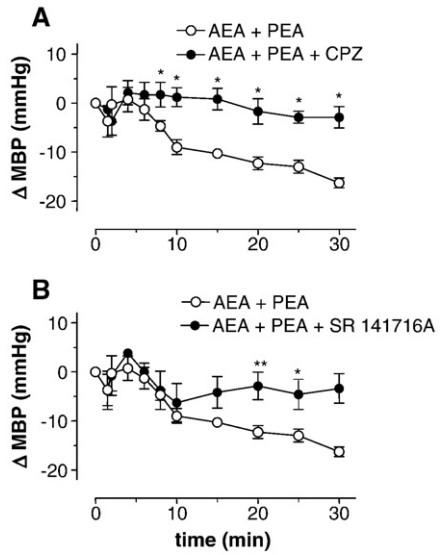

Fig. 4. Effects of capsazepine (CPZ) and SR 141716A on the decrease in mean blood pressure (ΔMBP; mmHg) induced by i.t. co-injection of 25 nmol anandamide (AEA) and 100 nmol palmitoylethanolamide (PEA). Filled symbols: either CPZ (20 nmol in A) or SR 141716A (20 nmol in B) was i.t. injected 5 min before time 0. Controls are depicted in open symbols. Shown are mean values ± S.E.M. of 4–5 animals per group. *$p<0.05$ vs. the corresponding control value.

As shown for anandamide, the metabolically stable analog methanandamide produced a hypotensive effect at the dose of 25 nmol only when it was co-injected with palmitoylethanolamide (Fig. 2A and D). Moreover, co-injection of palmitoylethanolamide enhanced the hypotensive response induced by 50 nmol methanandamide (Fig. 2B and E) but did not alter the response to 100 nmol (Fig. 2C and F).

A dose-dependent decrease in the mean blood pressure was also induced by the fatty acid derivative *N*-arachidonoyldopamine, i.e., significant decreases of about 12 and 17 mmHg were obtained with 50 and 100 nmol, respectively (Fig. 3B and C) whereas 25 nmol was devoid of effect (Fig. 3A). As observed for anandamide and methanandamide, when 25 nmol *N*-arachidonoyldopamine was co-injected with 100 nmol palmitoylethanolamide a significant hypotensive effect was produced (Fig. 3D). Co-administration of palmitoylethanolamide did not modify the blood pressure response induced by either 50 or 100 nmol *N*-arachidonoyldopamine (Fig. 3E and F).

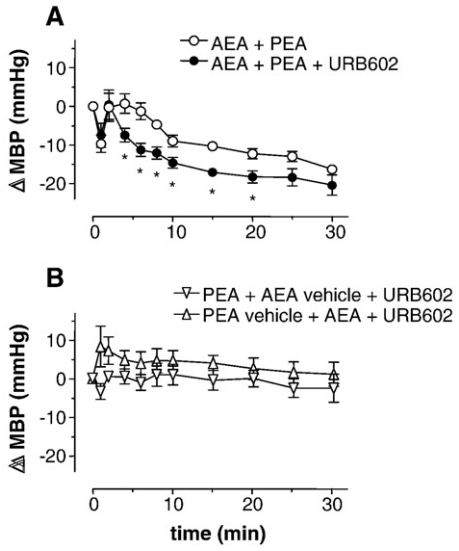

Fig. 5. Effects of URB602 on the decrease in mean blood pressure (ΔMBP; mmHg) induced by i.t. co-injection of 25 nmol anandamide (AEA) and 100 nmol palmitoylethanolamide (PEA). A: AEA was co-administered with PEA at time 0, either to control animals (open symbols) or to animals pretreated with URB602 (3.5 nmol; i.t.) 10 min before time 0 (filled symbols). B: Animals pretreated with URB602 received either PEA co-administered with AEA vehicle (10 μl 14% ethanol in saline) or PEA vehicle (10 μl 14% ethanol in 10% hydroxypropyl-β-cyclodextrin) co-injected with AEA. Shown are mean values ± S.E.M. of 4 animals per group. *$p<0.05$ vs. the corresponding control value. Vehicle (10 μl 14% ethanol in 10% hydroxypropyl-β-cyclodextrin) in A and AEA plus PEA in B.

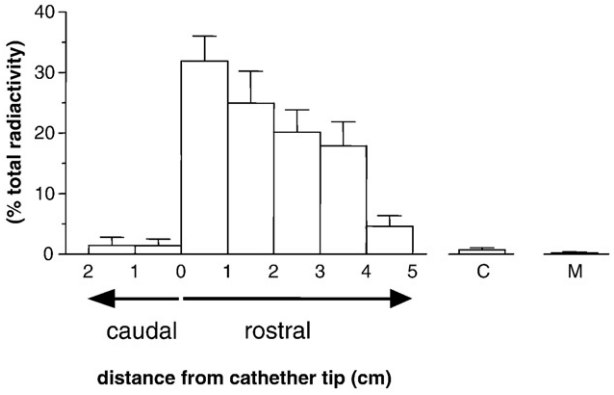

Fig. 6. Content of tritium in the bulbospinal axis 30 min after i.t. injection of 100 nmol [arachidonoyl-^{3}H]-anandamide (specific activity 0.19 mCi/mmol). The radioactivity per gram of wet tissue in the medulla oblongata (M), in the cervical spinal cord (C) and in the 1 cm segments of the spinal cord either caudal or rostral to the site of i.t. injection (distance 0) was expressed as a percentage of the total radioactivity present in the whole bulbospinal axis. Shown are mean values ± S.E.M. ($n=3$).

To analyze whether cannabinoid CB_1 and vanilloid TRPV1 receptors were involved in the blood pressure response caused by co-injection of anandamide and palmitoylethanolamide, rats were pretreated with either the TRPV1 receptor antagonist capsazepine (20 nmol; i.t.) or the CB_1 receptor antagonist SR 141716A (20 nmol; i.t.). As shown in Fig. 4, both antagonists completely prevented the hypotensive response caused by co-injection of palmitoylethanolamide with an ineffective dose of anandamide.

Opposite to the prevention of the effect of palmitoylethanolamide caused by the blockade of either CB_1 or TRPV1 receptors, an enhancement of the effect of palmitoylethanolamide was observed (Fig. 5A) when the experiments were performed in the presence of URB602 (3.5 nmol; i.t.), a putative inhibitor of palmitoylethanolamide degradation (Muccioli and Stella, 2008). In animals pretreated with URB602, neither 25 nmol anandamide nor palmitoylethanolamide modified the blood pressure *per se* (Fig. 5B). Capsazepine, SR 14171A and URB602 produced no changes in the baseline mean blood pressure (-4.5 ± 2.7 mmHg, 1.2 ± 1.9 mmHg and 4.2 ± 1.5 mmHg, respectively; $n=4$).

The heart rate of urethane anesthetized rats was not altered by the doses of agonists and antagonists assayed in this study (data not shown).

Thirty minutes after i.t. injection of 100 nmol [arachidonoyl-^{3}H]-anandamide, about 90% of the total radioactivity was found in the first 4 cm of the spinal cord rostral to the site of injection at T_{12}–L_1 level. The radioactivity was much lower in the lumbar segments as well as in the 4–5 cm segment that included the first thoracic levels, i.e. T_1–T_3. Negligible radioactivity was present both in the cervical region of the spinal cord and in the medulla oblongata (Fig. 6).

4. Discussion

This study shows that in urethane anesthetized rats the i.t. injection of the endocannabinoids *N*-arachidonoyldopamine and *N*-arachidonoylethanolamide (anandamide), as well as the i.t. administration of the non-hydrolyzable analog methanandamide, produced dose-dependent hypotensive effects that were enhanced by the

endogenous fatty acid derivative palmitoylethanolamide, considered to act as an entourage compound (De Petrocellis et al., 2001, 2002; Lambert and Di Marzo, 1999; Smart et al., 2002).

The baseline blood pressure values in the present report as well as in other studies performed on urethane anesthetized rats (García et al., 2003, 2006; Malinowska et al., 2001; Nakamura et al., 2007; Rochford et al., 1990) are lower than those reported for other anesthetics, namely urethane–chloralose mixture (Kopczyńska, 2007, 2008; Bertera et al., 2009). Nevertheless, we have previously demonstrated that the hypotensive effect of i.t. injected anandamide in urethane anesthetized rats does not change substantially when the baseline blood pressure is enhanced by intravenous infusion of phenylephrine (García et al., 2006).

We have also previously reported that the hypotensive effects of anandamide and methanandamide are related to the stimulation of both cannabinoid CB_1 and TRPV1 spinal receptors and may involve the release of calcitonin gene-related peptide (CGRP) and γ-aminobutyric acid (GABA) in the spinal cord (García et al., 2003, 2006). In agreement with this proposal, we have shown that the blockade of CGRP receptors and of either $GABA_A$ and $GABA_B$ receptors in the spinal cord prevent the hypotensive responses to i.t. injected anandamide (García et al., 2006). Moreover, i.t. injected CGRP and either $GABA_A$ and $GABA_B$ receptor agonists produce hypotension in urethane anesthetized rats (García et al., 1996, 2006). The hypotensive effects of *N*-arachidonoyldopamine probably involve the same spinal receptors and endogenous neurochemicals as those of anandamide, since *N*-arachidonoyldopamine is a CB_1 and TRPV1 receptor agonist (Sagar et al., 2004) and induces the release of CGRP (Huang et al., 2002) in the spinal cord.

The negligible radioactivity found in the medulla oblongata after i.t. injection of [arachidonoyl-^{3}H]-anandamide suggests that the i.t. injected compounds scarcely reached medullary cardiovascular areas. However, the possibility of a supraspinal effect, namely sympathoinhibition through the activation of presynaptic CB_1 receptors and inhibition of GABA release in the nucleus tractus solitary (Seagard et al., 2004, 2005) cannot be completely disregarded. On the other hand, the possibility that GABA release in the spinal cord may be negatively modulated through presynaptic CB_1 receptors cannot be ruled out, although at least in the dorsal horn, CB_1 receptors at GABAergic neurons seem to be localized in somas and dendrites exclusively (Salio et al., 2002).

The finding that the greatest effects of palmitoylethanolamide in the present study were observed with ineffective doses of anandamide, methanandamide and *N*-arachidonoyldopamine, which were four times lower than that of palmitoylethanolamide, agrees with the relatively high tissue content of endogenous palmitoylethanolamide compared to other fatty acid derivatives. In support of this view is the observation that the concentration of endogenous palmitoylethanolamide in the brain and in the spinal cord is at least three times higher than that of anandamide (Baker et al., 2001; Ferrer et al., 2007; Maccarrone et al., 2001; Maione et al., 2007; Petrosino et al., 2007). Moreover, very low tissue levels of *N*-arachidonoyldopamine have been detected in several brain nuclei and in dorsal root ganglia (Huang et al., 2002).

The observation that the hypotension induced by co-injection of palmitoylethanolamide with an ineffective dose of anandamide was prevented by either capsazepine or SR 144716A suggests that both TRPV1 and CB_1 receptors are involved in this response, as previously shown for the hypotensive response to anandamide (García et al., 2003). Since in human embryonic kidney cells palmitoylethanolamide enhances the influx of calcium induced by the activation of TRPV1 cation-channels by endocannabinoids (De Petrocellis et al., 2001, 2002), possibly through an allosteric effect of palmitoylethanolamide at TRPV1 receptors (De Petrocellis et al., 2001), we suggest that the facilitative effect of palmitoylethanolamide in the present study could be related to the enhancement of the TRPV1 receptor-mediated effects of endocannabinoids in the spinal cord. Nevertheless, this entourage effect indirectly affects spinal CB_1 receptors since the hypotensive effect of i.t. administered endocannabinoids requires the stimulation of both TRPV1 and CB_1 receptors (García et al., 2003 and present study).

It has also been suggested that palmitoylethanolamide enhances the effects of endocannabinoids such as anandamide by acting as a competitive inhibitor of the enzymatic degradation of endocannabinoids through the fatty acid amide hydrolase activity (Lambert and Di Marzo, 1999; Smart et al., 2002). However, it seems unlikely that a protective effect of palmitoylethanolamide against enzymatic hydrolysis of endocannabinoids could account for the effects of palmitoylethanolamide in our study. This is because the responses to anandamide, which is effectively hydrolyzed by the enzyme (Desarnaud et al., 1995; Maccarrone et al., 1998), and the responses to the metabolically stable analog methanandamide were similarly affected by palmitoylethanolamide.

Although some studies have reported that palmitoylethanolamide is degraded by the fatty acid amide hydrolase (Fowler et al., 2000; Tiger et al., 2000), there is evidence that palmitoylethanolamide is a better substrate for a distinct acid amidase (Tsuboi et al., 2005; Ueda et al., 2001). In addition, in microglia cells palmitoylethanolamide appears to be hydrolyzed by a novel enzyme that is inhibited by the carbamate compound URB602 (Muccioli and Stella, 2008). This evidence suggests that the potentiation of the entourage effect of palmitoylethanolamide by URB602 in the present study could be related to the prevention of the hydrolysis of palmitoylethanolamide in the spinal cord. To further analyze this possibility it should be necessary to determine whether a dose of palmitoylethanolamide higher than the one used in this study may produce a greater enhancement of the hypotensive responses to i.t. injected endocannabinoids; however, the low solubility of palmitoylethanolamide in aqueous vehicles prevented us from doing this experiment. On the other hand, URB602 is considered as a selective inhibitor of monoacylglycerol lipase, the enzyme that hydrolyses 2-arachidonoylglycerol (Hohmann et al., 2005). Since 2-arachidonoylglycerol is synthesized in the spinal cord (Baker et al., 2001; Maione et al., 2007; Petrosino et al., 2007), the participation of this endocannabinoid in the effect of URB602 cannot be completely precluded.

The observation that i.t injected endocannabinoids did not modify the heart rate as well as the finding of relatively low tritium content in the 4–5 cm segment of the spinal cord suggest that there was not an important diffusion of the compounds from the i.t. injection site at T_{12}–L_1 up to the T_1–T_3 level, where most of the preganglionic sympathetic neurons giving innervation to the heart are localized (Sundaram et al., 1989).

In summary, our findings suggest that palmitoylethanolamide acts as an entourage compound for the hypotensive responses to i.t. injected endocannabinoids in urethane anesthetized rats. The observation that palmitoylethanolamide principally enhanced the blood pressure responses to ineffective doses of endocannabinoids suggests that palmitoylethanolamide actually potentiates the effects to the usually low tissue levels of endocannabinoids.

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