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ORIGINAL ARTICLE

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Hypotensive effect of anandamide through the activation of CB_1 and VR_1 spinal receptors in urethane-anesthetized rats

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Abstract This study examined the effect of intrathecal (i.t.) injection of the endocannabinoid anandamide in urethane-anesthetized rats. The tip of the i.t. cannula was positioned at the T_{12}–L_1 level of the spinal cord. Either anandamide or its metabolically stable analogue methanandamide (25 to 100 nmol) produced dose-dependent decreases in the blood pressure that persisted at least for up to 30 min. The hypotensive responses to 100 nmol anandamide and to 100 nmol methanandamide were –17.7± 1.6 mmHg (n=5) and –17.9±2.0 mmHg (n=4), respectively. Hypotensive effects were also obtained with the CB_1 cannabinoid receptor agonist WIN 55212-2 (20 nmol; i.t.) as well as with the vanilloid VR_1 receptor agonist capsaicin (3 nmol; i.t.). Nicotinic ganglionic blockade with hexamethonium bromide [10 mg/kg; intravenous(i.v.)] abolished the responses to both anandamide and capsaicin. The i.t. administration of the CB_1 receptor antagonist, 20 nmol SR 141716A, as well as the VR_1 receptor antagonist, 20 nmol capsazepine, prevented almost completely the hypotensive responses to both anandamide and methanandamide. SR 141716A prevented the hypotension caused by WIN 55212-2 but did not modify the response to the vanilloid receptor agonist capsaicin. On the contrary, capsazepine antagonized the hypotension caused by capsaicin but failed to affect the decrease in blood pressure caused by the CB1 cannabinoid receptor agonist WIN 55212-2. These results suggest that anandamide could modulate the blood pressure through the activation of cannabinoid CB_1 receptors and vanilloid VR_1 receptors localized at the spinal cord.

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Introduction

Several of the complex cardiovascular effects caused by the endogenous cannabinoid anandamide, as well as by the plant-derived cannabinoid $^9\Delta$-tetrahydrocannabinol, appear to be related to the activation of peripheral sites of action. In this regard, the hypotension caused by intravenous (i.v.) administration of cannabinoids in anesthetized animals probably involves a decrease in sympathetic tone due to the activation of pre-synaptic cannabinoid CB_1 receptors in vascular and cardiac sympathetic nerve endings (Varga et al. 1996; Lake et al. 1997; Malinowska et al. 2001; Niederhoffer et al. 2003). Moreover, there is evidence that the hypotension induced by anandamide in spontaneously hypertensive rats involves the activation of vanilloid VR_1 receptors and the release of the potent vasodilator calcitonin gene-related peptide from perivascular sensory nerves (Li et al. 2003). On the other hand, anandamide produces reflex bradycardia and hypotension due to the activation of vanilloid VR_1 receptors in vagal (Malinowska et al. 2001) and perivascular (Smith and McQueen 2001) sensory nerves.

Some early studies proposed that, in addition to their peripheral actions, cannabinoids elicit cardiovascular responses by interaction with sites in the central nervous system (Cavero et al. 1973; Vollmer et al. 1974). In this regard, activation of cannabinoid CB_1 receptors in brainstem cardiovascular centers produces sympathoexcitation and bradycardia in conscious rabbits (Niederhoffer and Szabo 1999, 2000). Moreover, inhibitory effects of cannabinoids in the rostral ventrolateral medulla may contribute to the hypotension produced by their systemic injection in anesthetized rats (Niederhoffer et al. 2003).

Cannabinoid CB_1 receptors and vanilloid VR_1 receptors are expressed in the dorsal horn of the spinal cord, where they could have a role in the processing of nocicep-

tive stimuli (Szallasi and Blumberg 1999; Morisset et al. 2001; Szallasi and Di Marzo 2000; Di Marzo et al. 2002). In addition, the existence has been reported of CB_1 receptors in intermediolateral nuclei of the thoracic spinal cord (Farquhar-Smith et al. 2000), where pre-ganglionic sympathetic neurons are localized.

Since the sympathetic vasoconstrictor and cardiac tone largely depends on the activity of pre-ganglionic sympathetic neurons, the aim of the present study was to examine whether cannabinoids produce cardiovascular effects when administered at the spinal cord of urethane-anesthetized rats.

Materials and methods

Surgical procedures

The studies were conducted 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. 1997). Male Sprague–Dawley rats (250–300 g) were housed in groups of four in a room maintained at 21–23°C under a 12-h light–dark cycle. Food and water were freely available. The animals were anesthetized with urethane (1.2 g/kg; i.p.). The depth of anesthesia (surgical plane) was verified by the absence of the eyelid reflex. Although the animals breathed spontaneously, the trachea was cannulated to avoid respiratory disorders related to the accumulation of secretions in the superior airways. A polyethylene cannula was placed in the right femoral artery for recording of the blood pressure. For i.t. injection of drugs, a cannula was positioned at the level of the T_{12}–L_1 intervertebral space as described by Dib (1984). Briefly, a cannula (outside diameter 0.65 mm) was inserted into the subarachnoid space at the level of the C_8–T_1 vertebrae and gently pushed downward 4.5 cm. The position of the cannula was verified postmortem by direct observation after the ventral aspect of the vertebrae had been opened. 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 monitored by a rectal probe and was maintained at 37–38°C by a heating lamp.

Blood pressure recording and heart rate calculation

Blood pressure was measured from the right femoral artery via a Statham P23 1D transducer and recorded on a Grass 7B polygraph (Quincy, Mass., USA). 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. Baseline blood pressure and heart rate were recorded for at least 30 min before the experiment was started. 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.

Intrathecal injection of drugs

Drugs and vehicle solutions (10 µl) were intrathecally administered through a Hamilton microsyringe. An additional 10 µl of saline was injected to clear the catheter of the drug solution. The total injection of the drug plus saline lasted 1 min.

Experimental protocols

Animals were given a single dose of either anandamide (25, 50 or 100 nmol) or methanandamide (25, 50 or 100 nmol), or WIN 55212-2 (20 nmol) or capsaicin (3 nmol). The blood pressure and heart rate were recorded just before (time 0) and at the following times after the beginning of the i.t. injection: 1, 2, 4, 6, 8, 10, 15, 20, and 30 min. When indicated, the agonists were administered either 10 min after i.t. injection of the cannabinoid CB_1 receptor antagonist SR 141716A (20 nmol) or 5 min after i.t. injection of the VR_1 vanilloid receptor antagonist capsazepine (20 nmol). The choice of a shorter period of time for capsazepine than for SR141716A (5 min and 10 min, respectively) was made after we had taken into account the reportedly short duration of action of capsazepine (Lee and Lundberg 1994; Smith and McQueen 2001).

To analyze the effects of nicotinic ganglionic blockade on the blood pressure responses to either anandamide (100 nmol) or capsaicin (3 nmol), we injected the drugs, intrathecally, 5 min after i.v. injection of hexamethonium bromide (10 mg/kg). A continuous i.v. infusion of phenylephrine (0.2–0.3 µg/min) was started immediately after the bolus i.v. injection of hexamethonium bromide. The dose of phenylephrine for each animal was selected so as to attain baseline blood pressure values that did not differ from those observed before hexamethonium bromide injection. After that, the selected dose was maintained up to the end of the experiment.

Doses of drugs were selected on the basis of the following studies: phenylephrine (García et al. 1997), capsaicin and capsazepine (Ohkubo and Shibata 1997; Palazzo et al. 2002), and WIN 55212-2 and SR 141716A (Welch et al. 1998; Martin et al. 1999; Johanek et al. 2001).

Drugs

Anandamide and R-(+)-methanandamide were obtained from Cayman Chemical Co. (USA). Capsaicin, R-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethylmethanone mesylate (WIN 55212-2) and capsazepine were obtained 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). Hexamethonium bromide and phenylephrine HCl were purchased from Sigma Chemical Co. (USA).

Anandamide and R-(+)-methanandamide were supplied as ethanol solutions (50 mg/ml) and diluted with saline. Capsaicin and capsazepine were dissolved in ethanol and further diluted with saline. The final concentration of ethanol for the four drugs was 14% (v/v). WIN 55212-2 and SR 141716A were dissolved in 1% w/v solution of hydroxypropyl-β-cyclodextrin. Hexamethonium bromide and phenylephrine were dissolved in saline. Neither 14% ethanol nor 1% hydroxypropyl-β-cyclodextrin (intrathecally) modified baseline blood pressure values (Figs. 1 and 2).

Statistics

All values represent the mean ±SEM. Statistical differences were assessed by either unpaired Student's *t*-test or two-way analysis of variance for either independent or repeated measures. Post-hoc comparisons were performed with Newman–Keuls' test. *P* values smaller than 0.05 were regarded as significant.

Results

Cardiovascular responses induced by intrathecal injection of anandamide and methanandamide

In urethane-anesthetized rats, the resting mean blood pressure and the resting heart rate were 75.6±1.1 mmHg and 394±4 beats/min, respectively (n=153).

The i.t. administration of either the endocannabinoid anandamide or the metabolically stable analogue, meth-

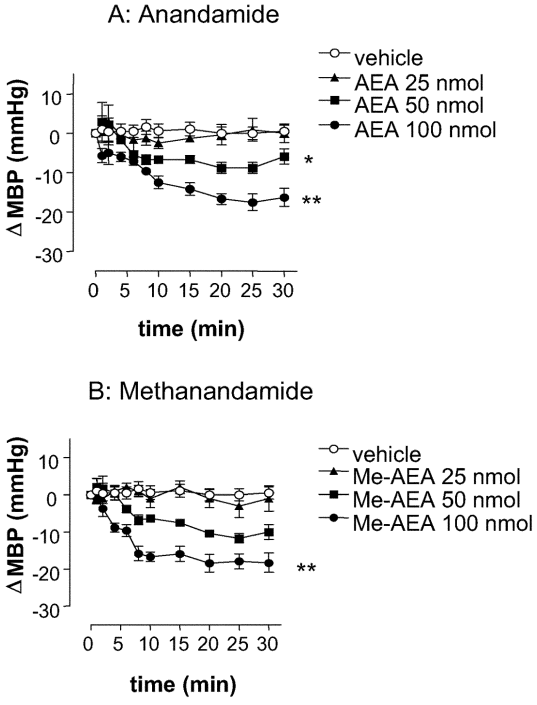

Fig. 1A, B Time-course of the decreases in the mean blood pressure (*ΔMBP*; mmHg) induced by i.t. injection of anandamide (*AEA*; **A**) and methanandamide (*Me-AEA*; **B**). The agonists or their vehicle (14% ethanol in saline) were injected in 1 min, starting at time 0. Shown are mean ± SEM for 4–5 animals per dose. **P*<0.05; ***P*<0.001 vs vehicle (two-way analysis of variance for repeated measures)

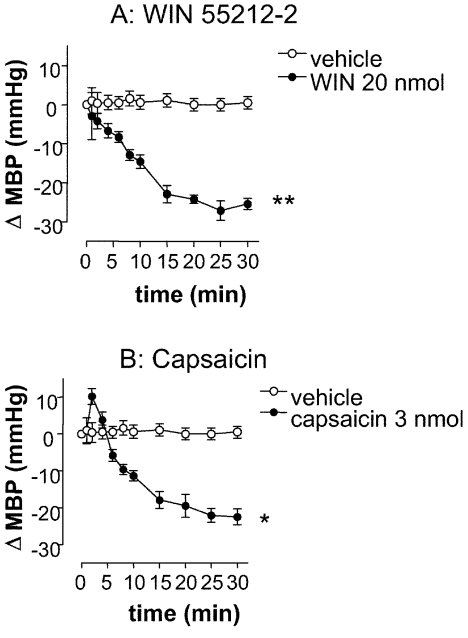

Fig. 2A, B Time-course of the decreases in the mean blood pressure (*ΔMBP*; mmHg) induced by i.t. injection of WIN 55212-2 (20 nmol; **A**) and capsaicin (3 nmol; **B**). The agonists or the corresponding vehicles (**A** 1% hydroxypropyl-β-cyclodextrin in distilled water; **B** 14% ethanol in saline) were injected in 1 min, starting at time 0. Shown are mean ± SEM for 4–5 animals per dose. **P*<0.05; ***P*<0.001 vs vehicle (two-way analysis of variance for repeated measures)

anandamide, induced a decrease in the mean blood pressure at doses of 50 and 100 nmol, whereas at a lower dose (25 nmol) they were devoid of effect. The decreases in the mean blood pressure were maximal 10–20 min after the beginning of the injections and persisted at least for up to 30 min (Fig. 1). Diminutions in the mean blood pressure were also obtained with the CB_1 receptor agonist WIN 55212-2 (Fig. 2A) as well as with the selective vanilloid VR_1 receptor agonist capsaicin (Fig. 2B). In the case of capsaicin, a small but significant increase in the mean blood pressure was observed 2 min after its i.t. injection (capsaicin 10.2±2.2 mmHg; vehicle 0.4±2.7 mmHg; $P<0.05$; $n=5$ each group).

Effects of the CB_1 receptor antagonist SR 141716A and the vanilloid receptor antagonist capsazepine, on the hypotensive responses induced by anandamide and methanandamide

The i.t. administration of the CB_1 receptor antagonist, 20 nmol SR 141716A, as well as the vanilloid receptor antagonist, 20 nmol capsazepine, prevented almost completely the hypotensive responses to both 100 nmol anandamide (Fig. 3A and B) and 100 nmol methanandamide (Fig. 3C and D).

As shown in Fig. 4A and C, 20 nmol SR 141716A also prevented the hypotensive effect induced by i.t. injection of the CB_1 receptor agonist WIN 55212-2 but did not modify the response to the vanilloid agonist, capsaicin. On the contrary, 20 nmol capsazepine antagonized the hypotension caused by capsaicin but failed to affect the decrease in mean blood pressure produced by WIN 55212-2 (Fig. 4B and D). In the animals pretreated with either capsazepine or SR 141716A, the changes in the blood pressure produced by capsaicin at an early stage, i.e., 2 min after injection, were rather inconsistent (5.9±4.4 mmHg and 4.2±2.3 mmHg, respectively; $n=6$ each group).

The change in the resting mean blood pressure induced by i.t. injection of either SR 141716A (2.3±2.1 mmHg; $n=13$) or capsazepine (4.1±1.9 mmHg; $n=13$) did not differ from those induced by i.t. administration of their corresponding vehicle solutions (2.3±2.1 mmHg and –3.4±1.8 mmHg, respectively; $n=5$ each group).

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

Effects of blockade of nicotinic ganglionic transmission on the cardiovascular responses to intrathecal injection of anandamide and capsaicin

To rule out the possibility that the blood pressure responses induced by intrathecally injected anandamide and capsaicin had resulted from peripheral effects due to leakage of the drugs from the site of injection, we analyzed their effects on mean blood pressure in rats pretreated with the ganglionic blocking agent hexamethonium bromide (10 mg/

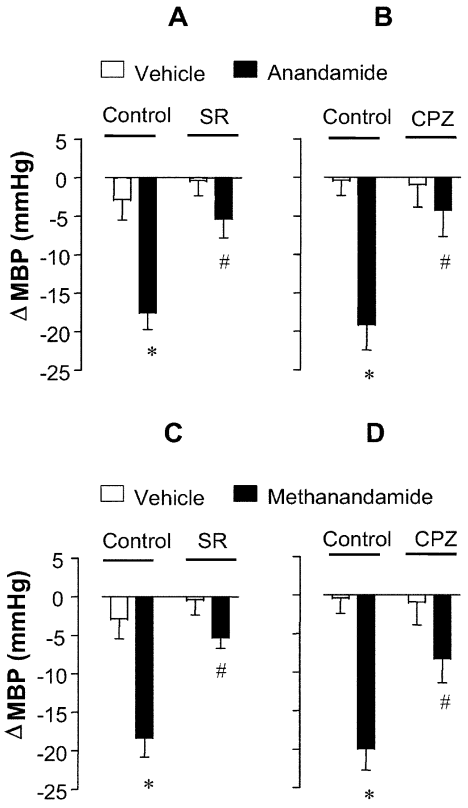

Fig. 3A–D Effects of SR 141716A and capsazepine on the blood pressure changes (*ΔMBP*; mmHg) induced by i.t. injection of either anandamide (100 nmol; **A** and **B**) or methanandamide (100 nmol; **C** and **D**). Anandamide, methanandamide, or the vehicle was intrathecally injected in 1 min, starting at time 0. Changes in MBP were measured 20 min after injection. The animals were pretreated with either SR 141716A (SR 20 nmol; i.t.) or capsazepine (*CPZ* 20 nmol; i.t.). In the control groups the animals were pretreated with 1% hydroxypropyl-β-cyclodextrin in distilled water (**A, C**) or with 14% ethanol in saline (**B, D**). Shown are mean values ± SEM for 5–6 sets of data. **P*<0.01 vs vehicle; #*P*<0.01 vs the corresponding value in the control group (two-way analysis of variance for independent measures followed by Newman–Keuls test)

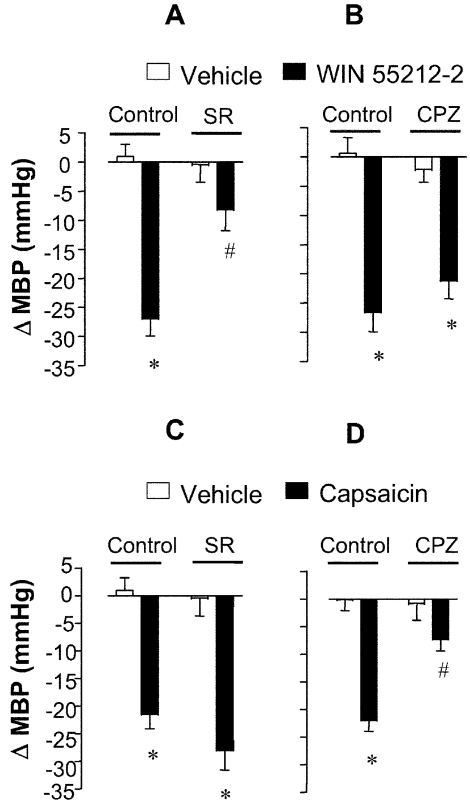

Fig. 4A–D Effects of SR 141716A and capsazepine on the blood pressure changes (*ΔMBP*; mmHg) induced by i.t. injection of either WIN 55212-2 (20 nmol; **A** and **B**) or capsaicin (3 nmol; **C** and **D**). WIN 55212-2, capsaicin, or the corresponding vehicles were intrathecally injected in 1 min, starting at time 0. Changes in MBP were measured 20 min after injection. The animals were pretreated with either SR 141716A (20 nmol; i.t.) or capsazepine (20 nmol; i.t.). In the control groups the animals were pretreated with 1% hydroxypropyl-β-cyclodextrin in distilled water (**A, C**) or with 14% ethanol in saline (**B, D**). Shown are mean values ± SEM for 5–6 sets of data. **P*<0.01 vs vehicle; #*P*<0.01 vs the corresponding value in the control group (two-way analysis of variance for independent measures followed by Newman–Keuls test)

Table 1 Effect of hexamethonium bromide on the maximal changes in mean blood pressure (ΔMBP) induced by intrathecal injection of anandamide and capsaicin. Shown are mean values ± SEM. *n* = number of animals

Experimental group	n	ΔMBP (mmHg)
Anandamide (100 nmol; i.t.)		
Control	5	–18.7±1.3
Hexamethonium[a]	4	–0.8±2.4*
Capsaicin (3 nmol; i.t.)		
Control	5	–24.3±2.5
Hexamethonium[a]	4	3.7±3.8*

**P*<0.001 vs the corresponding value in the control group (Student's *t*-test).

[a]Hexamethonium bromide (10 mg/kg; i.v.) was administered 5 min before the beginning of i.t. injection of either anandamide or capsaicin. A continuous i.v. infusion of phenylephrine (0.2–0.3 μg/min) was started immediately after the bolus i.v. injection of hexamethonium bromide and it was maintained up to the end of the experiment

kg; i.v.). To overcome the decrease in the baseline mean blood pressure that is usually caused by hexamethonium bromide, we gave the animals a continuous infusion of phenylephrine (0.2–0.3 μg/min). Under the latter conditions the baseline mean blood pressure value (69.2±4.0 mmHg; *n*=8) did not differ from that observed before hexamethonium bromide injection (69.8±3.9; *n*=8). Hexamethonium bromide abolished the hypotensive responses induced by both anandamide and capsaicin (Table 1) but did not modify the brief pressor response caused by capsaicin at the first 2 min post-injection (control 10.2±2.2 mmHg, *n*=5; hexamethonium bromide 10.8±2.9 mmHg, *n*=4).

Discussion

This study shows that i.t. injection of the endocannabinoid anandamide induces hypotensive effects in urethane-anesthetized Sprague–Dawley rats.

The decreases in blood pressure induced by i.t. injection of either anandamide or its metabolically stable analogue methanandamide were unlikely due to an action of the drugs on blood vessels after diffusion outside the central nervous system. This is because the response to anandamide was completely prevented by nicotinic ganglionic blockade with hexamethonium bromide. Furthermore, the hypotension produced by i.t injection of anandamide and methanandamide in this study was quite different from the cardiovascular responses induced by systemic injection of these drugs. As previously reported (Lake et al. 1997; Malinowska et al. 2001), i.v. administration of either anandamide or methanandamide to anesthetized rats produces a distinctive triphasic response that consists of an initial reflex bradycardia and transient drop in the blood pressure within 15 s (phase I), a brief pressor response during the following 30–150 s (phase II) and delayed hypotension that lasts 10 min, approximately (phase III). The transient reflex hypotension was also observed after intra-arterial injection of anandamide in anesthetized rats (Smith and McQueen 2001).

The finding that intrathecally injected agonists did not alter the heart rate in this study could indicate that there was not an important diffusion of the drugs from the injection site at T_{12}–L_1 toward the T_1–T_3 level, where the major part of the pre-ganglionic sympathetic neurons giving innervation to the heart are localized (Sundaram et al. 1989).

The hypotensive effects caused by anandamide and methanandamide in urethane-anesthetized rats appear to involve the activation of both CB_1 and VR_1 receptors in the spinal cord, because the responses to the agonists were prevented by i.t. administration of either the CB_1 receptor antagonist SR 141716A or the VR_1 receptor antagonist, capsazepine. This view is further supported by the observation that the CB_1 receptor agonist WIN 55212-2 and the VR_1 receptor agonist capsaicin produced hypotensive responses that were prevented by blockade of CB_1 and VR_1 receptors, respectively. The possibility of a non-specific action of the antagonists at the doses used in this study is ruled out on the basis that neither the VR_1 receptor antagonist modified the hypotensive response induced by the cannabinoid receptor agonist, nor did the CB_1 receptor antagonist modify the hypotension caused by the VR_1 receptor agonist.

Since, in this study, spinal cannabinoid and vanilloid receptors mediate the same kind of biological response, i.e., a decrease in blood pressure, it would be expected that the activation of either receptor by anandamide would make a partial contribution to the measured response. In fact, this occurs with the inhibitory effects of anandamide on electrically evoked contractions in the vas deferens (Ross et al. 2001) and with the anti-proliferative effects of anandamide in rat C6 glioma cells (Jacobsson et al. 2001). Nevertheless, the finding that the hypotensive responses to the agonists were completely prevented by selective blockade of either CB_1 or VR_1 spinal receptors rather suggests that intrathecally administered anandamide and methanandamide require the simultaneous participation of both CB_1 and VR_1 spinal receptors to produce hypotensive effects. By contrast, the experiments with WIN 55212-2 and capsazepine demonstrated that hypotensive responses can be obtained by selective activation of either CB_1 or VR_1 receptors. A tentative hypothesis to explain these observations is that the degree of activation caused by anandamide on a single type of spinal receptor, i.e., when one of them is blocked by an antagonist, could be inadequate to produce the necessary changes leading to measurable decreases in blood pressure. This may not occur with WIN 55212-2 and capsaicin, which are more potent than anandamide in activating the corresponding receptors (Terranova et al. 1995; Ross et al. 2001). It has been suggested that CB_1 and VR_1 receptors may act synergistically when activated by anandamide (Ross et al. 2001). Moreover, it was reported that activation of CB_1 receptors by anandamide can enhance the stimulation of VR_1 receptors by the same compound when both receptors are co-expressed in the same cell (Hermann et al. 2003). Therefore, the possibility of cross-talk between CB_1 and VR_1 spinal receptors involved in the central hypotensive effects of both anandamide and methanandamide cannot be ruled out.

This is in contrast to the hypotension caused by i.v. injection of anandamide in anesthetized animals, which appears to be entirely due to the activation of cannabinoid CB_1 receptors, as suggested by the observation that this response is abolished in CB_1 receptor knockout mice (Járai et al. 1999).

The brief pressor effect produced by capsaicin at the first 2 min post-injection was not clearly related to the activation of either VR_1 or CB_1 spinal receptors but probably involved the activation of a peripheral site of action, since it was not prevented by nicotinic ganglionic blockade. This pressor effect might be similar to the transient increase in blood pressure observed after i.v. injection of capsaicin or anandamide or methanandamide (Varga et al. 1996; Malinowska et al. 2001; Li et al. 2003), which is produced through a still unknown mechanism.

The localization of the CB_1 and VR_1 receptors in the spinal cord, and the neuronal pathways involved in the hypotensive effects of intrathecally administered cannabinoid and vanilloid agonists, remain unknown. A decrease in pre-ganglionic sympathetic neuron firing probably occurs because these neurons are the final station for the integration of central nervous system inputs into vasomotor nerves (Coote 1988).

Spinal CB_1 receptors were mostly detected in the dorsal horn, but it has been reported that CB_1 receptors are also expressed in the intermediolateral horn of thoracic segments, either in interneurons (Farquhar-Smith et al. 2000) or in cells that could be pre-ganglionic sympathetic neurons (Ong and Mackie 1999). Therefore, the possibility exists that the hypotensive responses observed in this study were related, at least in part, to a decrease in pre-ganglionic sympathetic nerve activity due to the stimulation of CB_1 receptors either in pre-ganglionic sympathetic neurons or in the surroundings neuronal pathways. CB_1 and VR_1 receptors expressed in the dorsal horn of the spinal cord (Tsou et al. 1998; Szallasi and Blumberg 1999;

Farquhar-Smith et al. 2000; Morisset et al. 2001) may be involved in the modulation of nociceptive transmission (Welch et al. 1998; Richardson et al. 1998; Szallasi and Di Marzo 2000; Morisset et al. 2001). Since there is evidence of interactions between nociceptive pathways and preganglionic sympathetic neurons (Rozsa et al. 1988; Chau et al. 2000; Minson et al. 2002), the possibility that the hypotensive responses induced by anandamide in this study were related to the activation of CB_1 and VR_1 receptors in the dorsal horn cannot be disregarded.

Although anandamide and other endogenous cannabinoids are synthesized in the spinal cord (Di Marzo et al. 2000; Fezza et al. 2002), CB_1 and VR_1 receptors involved in the hypotensive effects observed here do not appear to be tonically activated in the present experimental conditions in so far CB_1 and VR_1 receptor blockade in the spinal cord did not alter baseline blood pressure values.

Whereas the ability of anandamide to activate VR_1 receptors in vitro is well documented (Di Marzo et al. 2002), the information about in vivo effects of anandamide linked to the activation of VR_1 receptors is scarce (see Malinowska et al. 2001; Jia et al. 2002 and Li et al. 2003 for examples). In this regard, the present work showing that i.t. administration of anandamide induces hypotensive effects through the activation of both CB_1 and VR_1 spinal receptors is a novel observation and may represent a clue for future studies.

In conclusion, the present results in urethane-anesthetized rats suggest that anandamide could modulate the blood pressure not only through the interaction with peripheral sites but also through the activation of CB_1 and VR_1 receptors at the spinal cord.

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