Naunyn-Schmied Arch Pharmacol (2010) 381:349–360
DOI 10.1007/s00210-010-0497-6

ORIGINAL ARTICLE

Involvement of central β_2-adrenergic, NMDA and thromboxane A_2 receptors in the pressor effect of anandamide in rats

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Received: 9 January 2010 / Accepted: 6 February 2010 / Published online: 3 March 2010

Abstract Intravenous (i.v.) injection of the endocannabinoid anandamide induces triphasic cardiovascular responses, including a pressor effect mediated via unknown central and peripheral mechanism(s). The aim of the present study was to determine the central mechanism(s) responsible for the pressor response to anandamide. For this purpose, the influence of antagonists at thromboxane A_2 TP (sulotroban, daltroban, SQ 29548), NMDA (MK-801) and β_2-adrenergic receptors (ICI 118551) on the pressor effect induced by i.v. and intracerebroventricularly (i.c.v.) administered anandamide was examined in urethane-anaesthetized rats. Anandamide (1.5–3 μmol/kg, i.v.) or its stable analogue methanandamide (0.75 μmol/kg, i.v.) increased blood pressure by 25%. Anandamide (0.03 μmol per animal i.c.v.) caused a pure pressor effect (by 20%) but only in the presence of antagonists of CB_1 and TRPV1 receptors. The effects of cannabinoids (i.v. or i.c.v.) were diminished by i.v. daltroban, sulotroban (10 μmol/kg each), and/or SQ 29548 (1 μmol/kg). The effect of anandamide i.v. was reduced by SQ 29548 (0.02 μmol per animal i.c.v.) and by the thromboxane A_2 synthesis inhibitor furegrelate i.c.v. (1.8 μmol per animal). ICI 118551, MK-801 (1 μmol/kg i.v. each), and bilateral adrenalectomy diminished the effect of anandamide i.c.v. Sulotroban (i.v.) failed to affect the response to anandamide (i.v.) in pithed rats, and anandamide and methanandamide did not bind to TP receptors in rat platelets. The present study suggests that central β_2-adrenergic, NMDA and thromboxane A_2 receptors are involved in the anandamide-induced adrenal secretion of catecholamines and their pressor effect in urethane-anaesthetized rats.

Keywords Anandamide · β_2-adrenoceptors · Cannabinoid receptors · NMDA receptors · Thromboxane A_2 receptor · TRPV1 receptor

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Introduction

Anandamide is one of the endogenous ligands of the endocannabinoid system and plays an important role under various physiological and pathophysiological conditions (for review, see Di Marzo 2009). In the cardiovascular system, anandamide elicits both hypo- and hypertensive responses and acts both via cannabinoid receptor-dependent and receptor-independent sites (for review, see Pacher et al. 2008). In order to disclose its numerous facets of cardiovascular effects, anaesthetized rodents are used frequently. Intravenous (i.v.) injection of anandamide to anaesthetized normotensive rats (Varga et al. 1995; 1996; Lake et al. 1997; Malinowska et al. 2001a; Kwolek et al. 2005) and mice (Pacher et al. 2004) causes a triphasic

cardiovascular response, namely an initial rapid and short-lasting bradycardia and hypotension, known as the Bezold-Jarisch reflex (phase I), a subsequent increase in blood pressure (phase II), and a delayed and prolonged hypotension (phase III). The mechanisms underlying phases I and III have been clarified. Thus, phase I involves the activation of vanilloid TRPV1 receptors located on sensory vagal nerves in the heart (Malinowska et al. 2001a). Phase III involves several mechanisms including the activation of (1) presynaptic cannabinoid CB_1 receptors innervating the sympathetic neurones supplying blood vessels and heart (Malinowska et al. 1997, 2001b; Niederhoffer et al. 2003), (2) of CB_1 receptors causing a decrease in cardiac contractility (Bátkai et al. 2004), (3) of TRPV1 receptors in the spinal cord (del Carmen Garcia et al. 2003), and (4) of non-CB_1 vascular receptors sensitive to O-1918 (Zakrzeska et al. 2010). Unlike in the anaesthetized rat, anandamide induces only the initial phase I and the subsequent pressor response in conscious rats (Lake et al. 1997; Gardiner et al. 2009).

The mechanisms underlying the hypertensive effect, i.e. phase II, of the triphasic response to anandamide in the anaesthetized rat, have not been fully disclosed. In experiments in urethane-anaesthetized rats, we found (Kwolek et al. 2005) that reflex- and CB_1 receptor-independent peripheral and central components are involved in the induction of this phase. One component is located most probably in blood vessels (Kwolek et al. 2005) and was not further considered in the present paper. A second component, which involves the central nervous system, was inhibited by the non-selective β-adrenoceptor antagonist propranolol, the β_2-adrenoceptor antagonist ICI 118551, and the non-selective NMDA receptor antagonist MK-801. In continuation of our previous work (Kwolek et al. 2005), this central mechanism of action was studied in more detail in the present work.

In particular, a prostanoid system capable of increasing blood pressure but not yet investigated in the context of the anandamide-induced pressor effect is the thromboxane A_2 (TXA_2)-TP receptor system. TXA_2 is one of the most potent peripheral vasoconstrictors and aggregators of thrombocytes (e.g. Sellers and Stallone 2008) and increasing evidence suggests that TXA_2 acts also as central neuromodulator of cardiovascular function (Gao et al. 1997; Murakami et al. 2002; Okada et al. 2000, 2008). Therefore, the first step of the present study was to incorporate experiments with TP receptor antagonists into the same scheme as applied with the drugs studied previously (Kwolek et al. 2005). Accordingly, we decided to examine in intact and pithed rats whether TP receptor antagonists inhibit the anandamide-induced pressor response. In fact, this response was diminished by TP receptor antagonists in intact, but not in pithed rats, suggesting a central site of action. Therefore, in a second step, we studied the underlying central mechanism in more detail by intracerebroventricularly injecting anandamide and appropriate pharmacological tools such as relevant receptor antagonists. In addition, radioligand binding experiments were carried out in order to study whether anandamide directly interacts with the TP receptor.

Methods

Experiments on whole animals

Male Wistar normotensive rats weighing 220–300 g were used in the present experiments. The animals were maintained at a 12/12 h light–dark cycle and housed in a special room at constant temperature (22±2°C) and humidity (50%) and had free access to water and standard rat chow. All surgical procedures and experimental protocols were approved by the local Animal Ethics Committee in Białystok (Poland). They have been carried out in accordance with the Guide for the Care and Use of Laboratory Animals.

Surgical procedure preparing for intracerebroventricular drug administration

Animals were anaesthetized intraperitoneally (i.p.) with pentobarbitone sodium (300 μmol/kg). They were prepared for intracerebroventricular injections exactly as described earlier (Braszko et al. 1991). Briefly, a circular piece of skin, 7 mm in diameter, was cut off the scalp, and the underlying skull surface was cleaned from soft tissue. A burr hole, 0.5 mm in diameter, was drilled in the skull 2.5 mm laterally and 1 mm caudally from the bregma on the right side of the head. The operation took about 2 min. Animals were housed in separate cages until the wound was completely dry, and the animal behaved normally. Intracerebroventricular injections were made freehand into the right cerebral ventricle with a 10-μl Hamilton syringe, using a KF 730 needle cut 4.5 mm from its base. This procedure allowed the tip of the needle to be lowered about 0.5 mm below the ceiling of the lateral cerebral ventricle. The injection volume was 2 μl per animal (or 5 μl per animal in the case of furegrelate), administered over 3 s. Upon completion of each experiment, rats were sacrificed and the sites of injections were verified microscopically after brain sectioning.

Anaesthetized rats

At least 72 h later, rats were anaesthetized i.p. with urethane (14 mmol/kg). The trachea was cannulated. Mean, systolic and diastolic blood pressure (MBP, SBP and DBP, respectively) were measured from the right carotid artery via a transducer (ISOTEC; Hugo Sachs Elektronik, March–Hugstetten,

Germany). We have mainly concentrated on the examination of DBP since this parameter reflects changes in vascular resistance. Moreover, i.v. and i.c.v. injection of anandamide and methanandamide induces more marked increases in DBP when compared with MBP and SBP (see Table 1 in Zakrzeska et al. 2010 and Fig. 1 in the present paper, respectively). Heart rate (HR) was measured by a rate–meter triggered from the pressure record. The left femoral vein was cannulated for i.v. injection of drugs administered in a volume of 0.5 ml/kg. Since the extent of vasopressor/vasodepressor effects is dependent on the basal level of DBP (Malinowska and Schlicker 1993), vasopressin (0.04–0.4 IU kg/min) was infused into the right femoral vein in some animals to have a DBP of 55–70 mmHg in each animal (vasopressin administration was necessary in all pithed (see below) and bilaterally adrenalectomized rats and in some intact anaesthetized animals). Body temperature was kept constant at about 37–38°C using a heating pad (Bio-Sys-Tech, Białystok, Poland) and monitored by a rectal probe (Physitemp BAT10, Clifton, NJ, USA). After surgical procedures, animals were gently placed on their abdomen. Fifteen to 30 min later, during which the cardiovascular parameters were allowed to stabilise, experiments were performed.

Pithed rats

Rats were anaesthetized i.p. with urethane (14 mmol/kg) and then injected i.p. with atropine (2 μmol/kg). After cannulation of the trachea, the animals were pithed by inserting a stainless-steel rod (1.5 mm diameter and 190 mm length) through the right orbit and the foramen magnum and down to the vertebral canal. Artificial respiration (1 ml/100 g, 60 strokes/min) with room air was immediately started using a respirator (7025 Rodent respirator, Hugo Sachs Elektronik, March–Hugstetten, Germany). Both vagal nerves were cut. Blood pressure, heart rate and body temperature were measured as described above. After 30 min of equilibration, during which the cardiovascular parameters were allowed to stabilise, experiments were performed.

Experimental protocol

Anandamide or methanandamide was injected twice i.v. (S_1 and S_2, 15 min apart) or only once i.c.v. (S_1) (anandamide only). Since individual differences in responses to anandamide were noticed, we applied i.v. anandamide at doses of 1.5–3 μmol/kg. We have chosen a dose of anandamide that increased DBP during phase II by about 20–30% of the basal value. Methanandamide was administered i.v. at 0.75 μmol/kg and anandamide i.c.v. at 0.03 μmol per animal. The TP receptor antagonists sulotroban (10 μmol/kg, i.v., Stegmeier et al. 1984), daltroban (10 μmol/kg, i.v., Bertolino et al. 1997) and SQ 29548 (1 μmol/kg, i.v., Bertolino et al. 1997 or 0.02 μmol per animal i.c.v., Yalcin et al. 2005), the NMDA receptor antagonist MK-801 (1 μmol/kg, i.v., Kwolek et al. 2005) and the β_2-adrenoceptor antagonist ICI 118551 (1 μmol/kg, i.v., Kwolek et al. 2005) or their solvents were administered i.v. or i.c.v. 5 min before the second (S_2) or the only (S_1) dose of anandamide (i.v. or i.c.v.) or methanandamide (i.v.),

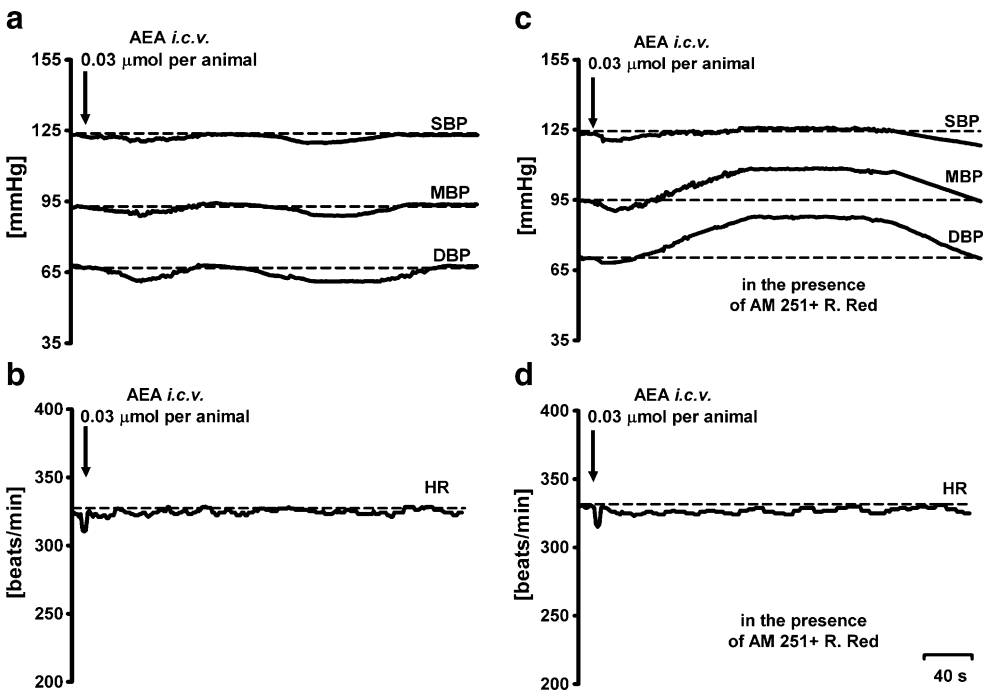

Fig. 1 Typical traces showing the changes in systolic (SBP), mean (MBP), diastolic (DBP) blood pressure and heart rate (HR) induced by i.c.v. injection of anandamide (AEA) in a urethane-anaesthetized rat **a**, **b** without and **c**, **d** after intravenous (i.v.) injection of AM 251 plus ruthenium red (AM 251+R. Red; 3 μmol/kg each, given 5 min before AEA). Note that the short-lived decrease in HR shown in panel **b** and **d** also occurred when the solvent for anandamide was used. *Arrows* indicate drug application

respectively. All experiments with i.c.v. administration of anandamide were performed in the presence of AM 251 (3 μmol/kg, Baranowska et al. 2008) and ruthenium red (3 μmol/kg, Malinowska et al. 2001a), given i.v. 5 min before anandamide i.c.v. AM 251 is a selective CB_1 receptor antagonist; ruthenium red is a non-selective TRPV1 receptor antagonist but was preferred over the selective TRPV1 receptor antagonist capsazepine due to its more marked and much longer antagonistic effect in the anaesthetized rat (Malinowska et al. 2001a). There are two exceptions from the above protocol. The inhibitor of thromboxane A_2 synthase, furegrelate (1.8 μmol per animal, Okada et al. 2008), was given i.c.v. 10 min after S_1 and 30 min before S_2. In some experiments, bilateral acute adrenalectomy or a sham operation was performed at the end of other surgical preparations described in the part "Anaesthetized rats." These rats received intramuscular (i.m.) injections of cortisol 3 μmol/kg or its solvent (250 μl saline per animal) together with anaesthesia (according to Okada et al. 2008).

Binding studies

Binding studies were carried out according to the method described by Hedberg et al. (1988) (modified). Wistar rats (Charles River, Sulzfeld, Germany) were killed by an overdose of 120 mg/kg pentobarbitone i.p. Blood was withdrawn from the vena cava by venipuncture and collected in a tube containing K-EDTA (1.2–2 mg EDTA/ml blood; Sarstedt, Nümbrecht, Germany). Needles (Sarstedt) were heparinised (heparin 25,000 IE/ml) prior to use to prevent immediate clotting. The tube was centrifuged immediately at 200×*g* for 20 min. The pellet was discarded, the supernatant recentrifuged at 1,000×*g* for 15 min, and the platelets (pellet) were resuspended gently in platelet buffer (concentration in millimolars: NaCl 145, HEPES 10, Na_2HPO_4 500, KCl 10, $MgCl_2$ 4, glucose 10, bovine serum albumin 45 μM). After centrifugation at 1,000×*g* for 15 min, the supernatant was discarded and the washing procedure was repeated in Tris-saline (50 mM Tris, 154 mM NaCl, pH 7.4). The resulting pellet was resuspended in Tris-saline to a final concentration of 50–80 μg/100 μl protein ($90–150\times10^6$ platelets/100 μl). Fresh cell suspension was used for receptor binding assays. Protein content was determined using the method of Bradford; cell count was determined in a Neubauer chamber. The binding assay was performed in Tris-saline buffer in a final volume of 0.5 ml containing 50–80 μg protein. ^{3}H-SQ 29,548 was used at eight concentrations ranging from 0.15 to 30 nM for saturation experiments and at a concentration of 3 nM for displacement experiments. The incubation was terminated after 30 min by filtration through polyethyleneimine (0.3%)-pretreated Whatman GF/C filters (Whatman, Maidstone, UK). All steps were carried out at room temperature. Unspecific binding was determined in the presence of 50 μM unlabeled SQ 29,548.

Calculations and statistics

Results are given as mean±standard error of the mean (SEM); *n* refers to the number of rats (whole animal experiments) and to the number of separate experiments in triplicate (binding studies). In order to quantify the effects of antagonists on the anandamide- and methanandamide-induced changes in cardiovascular parameters, S_1 and S_2 values were calculated as percent of the basal diastolic blood pressure immediately before injection of that particular agonist dose. In the case of two administrations of agonists (S_1 and S_2), the final results are "S_2" expressed as a percentage of S_1. For comparison of the mean values, the *t* test for paired and unpaired data was used, as appropriate. When two or more groups were compared with the same control, the one-way analysis of variance (ANOVA) followed by the Dunnett test was used. Differences were considered as significant when $P<0.05$. Radioligand binding curves were analysed by nonlinear curve fitting using the GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA).

Drugs used

AM 251 [*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide]; anandamide; (*R*)-(+)-methanandamide; ICI 118551 (erythro-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol; Tocris Cookson, Bristol, UK); atropine sulphate; ruthenium red; urethane; [Arg^8]-vasopressin; daltroban; cortisol; MK-801 ((5*R*,10 *S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo(a,d) cyclohepten-5,10-imine hydrogen maleate; Sigma, München, Germany); furegrelate (sodium salt; Cayman Chemical, Ann Arbor, MI, USA); U 46,619 (9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$; Biomol, Hamburg, Germany); SQ 29,548 ([1 S-[1α, 2α(Z), 3α, 4α]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; Cayman or Biomol); pentobarbitone sodium (Biowet, Puławy, Poland or Abbott, Ludwigshafen, Germany); sulotroban (Boehringer Mannheim, Mannheim, Germany); heparin sodium (ratiopharm, Ulm, Germany); ^{3}H-SQ 29,548 (specific activity 43.6 Ci/mmol; PerkinElmer, Boston, MA, USA).

Drugs used for the whole animal experiments were dissolved in saline with the following exceptions: AM 251 in a mixture of ethanol, Cremophor El, DMSO and saline (1:1:1:9.5); daltroban in a mixture of 2 mM Na_2CO_3 and 2 mM NaOH (50:1); sulotroban in a mixture of saline (0.9% NaCl) and 2 M NaOH (50:1); SQ 29548 in a mixture of saline and DMSO (20:1); cortisol in a mixture of $CHCl_3$

and ethanol (1:1). Anandamide and methanandamide were purchased from Tocris Cookson as 10 mg/ml emulsion in soya water (1:4). Vasopressin was provided by the manufacturer as an aqueous stock solution (100 IU/ml), which subsequently was diluted (1:74) in isotonic saline before the experiment. Intravenous injection of saline or the solvents for sulotroban, daltroban and SQ 29548 first decreased and then increased DBP by about 10–30% each; the alterations were short-lived (maximally by about 30 s). Basal HR was not affected. Intracerebroventricular administration of solvents did not change basal cardiovascular parameters, with the exception of a slight and short-lived decrease in HR. The drugs used for the binding studies were dissolved in ethanol (SQ 29548, U-46,619) or in ethanol plus bovine serum albumin 0.5% (anandamide, methanandamide). The solvents did not affect binding by themselves.

Results

Experiments on whole animals

In urethane-anaesthetized rats, the basal diastolic blood pressure measured immediately before the administration of the first (or only one) dose of agonist was in the range of 55–70 mmHg in most anaesthetized rats or was brought to this level by vasopressin in all pithed and adrenalectomized rats and in some intact anaesthetized animals (for the exact values, see later Figs. 3, 4, 5, 6 and 7; bottom of the columns). Basal HR was 369±6 (n=123), 357±9 (n=9), 349±30 (n=5) and 341±9 (n=6) beats/min in intact, pithed, bilaterally adrenalectomized and sham-operated rats, respectively. Antagonists (given i.v. or i.c.v.) did not affect basal DBP or HR.

Influence of intravenous and intracerebroventricular administration of anandamide and/or methanandamide on blood pressure

Intravenous injection of anandamide (1.5–3 μmol/kg) induced typical triphasic changes in cardiovascular parameters in urethane-anaesthetized rats as described by us in detail earlier (for typical traces see Fig. 1 in Kwolek et al. 2005; Malinowska et al. 2001a; Zakrzeska et al. 2010). Thus, the initial phase I, which consisted of a fall in SBP, MBP, DBP and HR, was followed by an increase in blood pressure (phase II) and a prolonged hypotension (phase III). In pithed rats, only phase II and III occurred. In the present study on intact and pithed anaesthetized rats, anandamide or methanandamide were applied i.v. at doses that increased DBP by about 20–30% of basal values, i.e. anandamide 1.5–3 μmol/kg or methanandamide 0.75 μmol/kg. The same dose of each agonist was given twice (S_1 and S_2), and the increase in DBP was identical for S_1 and S_2 when the solvents for the antagonists under study were administered between both agonist injections (see columns in the absence of antagonists in Figs. 3, 4 and 6).

When anandamide (0.03 μmol per animal) was administered intracerebroventricularly, two hypotensive responses were noticed. Thus, after an initial, rapid (13±3 s; n=4) decrease in DBP, blood pressure returned to the basal value for about 20 s and then a longer (66±28 s; n=4) fall in DBP was observed (for original tracings, see Fig. 1a; for statistic evaluation, see Fig. 2a). An entirely different response to i.c.v. injection of anandamide (0.03 μmol per animal) was observed in the presence of the vanilloid receptor antagonist ruthenium red (3 μmol/kg) and the CB_1 receptor antagonist AM 251 (3 μmol/kg) given i.v. 5 min earlier. As shown in Figs. 1c and 2a, anandamide (0.03 μmol per animal) under this experimental condition produced a pure pressor effect (by about 20% of the basal value), which lasted for 228±59 s. Anandamide (0.03 μmol per animal) also induced a similar (but smaller) decrease (Fig. 1a) and increase (Fig. 1c) in MBP in the absence and the presence of simultaneous administration of ruthenium red and AM 251, respectively. However, it had only a marginal effect on SBP (Fig. 1a, c) and failed to affect HR (Fig. 1b, d) under both experimental conditions. The solvent of anandamide did not affect DBP, MBP and SBP but caused a short-lived decrease in HR (not shown) identical to that obtained with anandamide (Fig. 1b, d). In all further experiments, we have concentrated on changes in DBP.

In the experiments in which one of the ligands (agonist or antagonist) under study was injected by the i.c.v. route, ruthenium red plus AM 251 (3 μmol/kg, each) were given routinely i.v. 5 min beforehand (Figs. 2, 5, 6 and 7). The increase in DBP induced by i.c.v. administration of anandamide was dose-dependent (0.01–0.1 μmol per animal). The maximal effect (about 20% of the basal value) was obtained for the two highest doses (0.03 and 0.1 μmol per animal; Fig. 2b). Thus, in all further experiments anandamide was given i.c.v. at a dose of 0.03 μmol per animal (Figs. 5 and 7). The solvents of the antagonists under study (see below) did not affect the response to i.c.v. administration of anandamide 0.03 μmol per animal (increase by 20–25% of the basal value; see control values in Figs. 5 and 7).

Influence of thromboxane A_2 receptor antagonists and furegrelate on the pressor response to anandamide and methanandamide

The influence of three TP receptor antagonists sulotroban (10 μmol/kg), daltroban (10 μmol/kg) and SQ 29548 (1 μmol/kg) on the pressor response to anandamide was examined after its i.v. and i.c.v. administration. We found

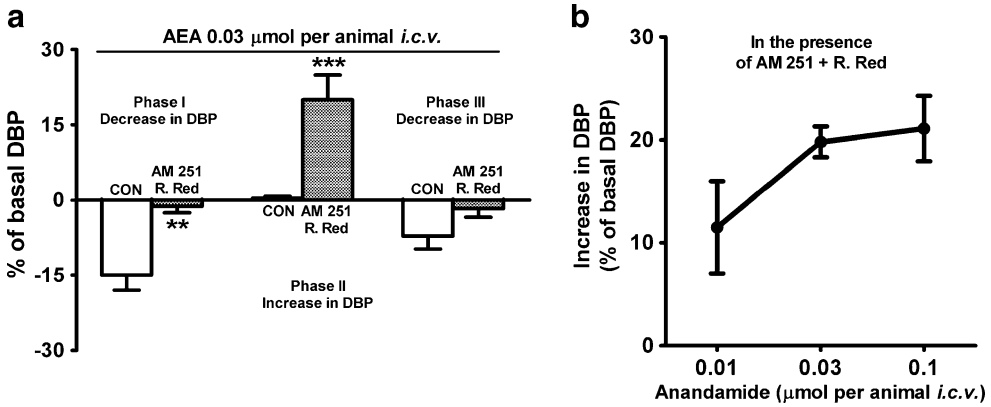

Fig. 2 Alteration of diastolic blood pressure (DBP) induced by i.c.v. administration of anandamide (AEA) in urethane-anaesthetized rats. Anandamide was given 5 min after combined intravenous (i.v.) injection of AM 251 and ruthenium red (AM 251+R. Red; 3 μmol/kg, each) or their solvents. Each dose of anandamide was studied in a separate rat. Panel **a** shows that anandamide 0.03 μmol per animal elicits a monophasic increase in DBP in rats treated with AM 251+R. Red and an early and delayed decrease in DBP in rats treated with their solvents instead. Panel **b** shows the dose-response curve for the anandamide-induced increase in DBP. Mean ± SEM of 4 (panel **a**) or of 3, 36 and 4 rats for 0.01, 0.3 and 0.1 μmol anandamide per animal, respectively (panel **b**). $^{**}P<0.01$, $^{***}P<0.001$ compared with the corresponding control (*CON*)

that in intact anaesthetized rats, i.v. administration of sulotroban reduced by about 35%, the increase in DBP induced by i.v. injection of anandamide and methanandamide (Figs. 3a, b). In contrast, in pithed animals, sulotroban i.v. failed to affect the pressor influence of anandamide i.v. (Fig. 3c). Thus, all further experiments were performed only in intact anaesthetized rats.

The increase in DBP elicited by anandamide i.v. was also inhibited by i.v. administration of another two TP receptor antagonists, i.e. daltroban and SQ 29548, by about 50 and 35%, respectively (Fig. 4). All further experiments were performed in the presence of ruthenium red plus AM 251 (3 μmol/kg, each). The i.v. injection of sulotroban, daltroban and SQ 29548 also reduced the effect of i.c.v. administered anandamide 0.03 μmol per animal by about 50%, 40% and 45%, respectively (Fig. 5). In the next set of experiments, anandamide 3 μmol/kg was given i.v. and SQ 29548 0.02 μmol per animal i.c.v. Again, the TP receptor antagonist effectively, by about 30%, reduced the pressor response to the endocannabinoid (Fig. 6a). The inhibitor of thromboxane A_2 synthase, furegrelate 1.8 μmol per animal i.c.v., was used to further examine the potential involvement of TXA_2 in the increase in DBP induced by i.v. administration of anandamide 3 μmol/kg. In fact, the

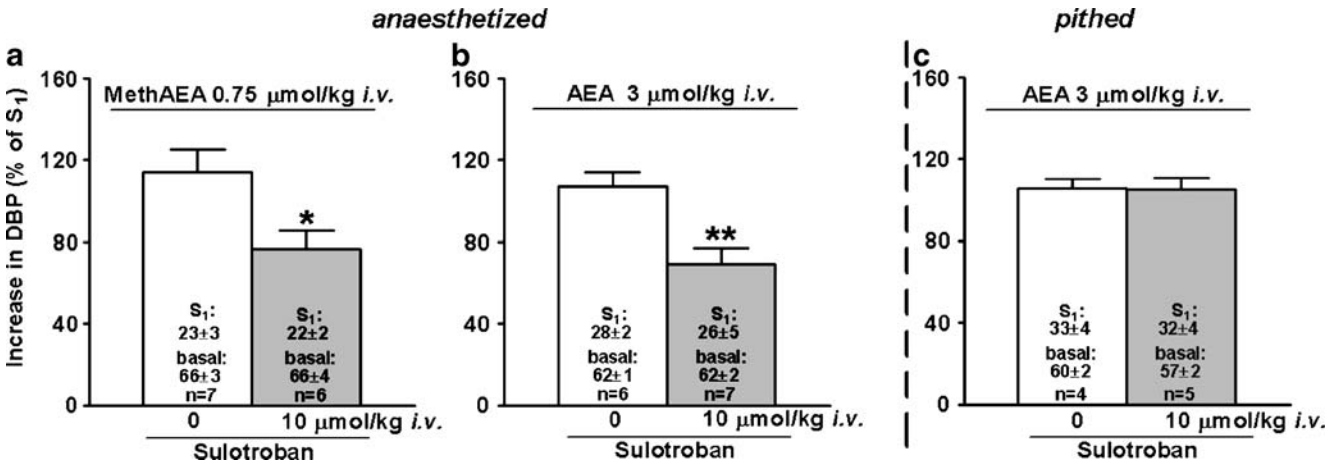

Fig. 3 Influence of intravenous (i.v.) injection of sulotroban on the increases in diastolic blood pressure (DBP) induced by i.v. administration of **a** methanandamide (MethAEA) or **b** anandamide (AEA) in urethane-anaesthetized intact rats or **c** in pithed and bilaterally vagotomized rats. AEA or MethAEA was injected twice (S_1 and S_2, 15 min apart). Sulotroban or its solvent was administered 5 min before S_2. S_1 and S_2 were calculated as percent of the respective basal values (values of S_1 and basal DBP (in millimetres of Hg) determined immediately prior to S_1 are given on *bottom of the columns*). Mean ± SEM of four to seven rats. $^{*}P<0.05$, $^{**}P<0.01$, compared with the corresponding control

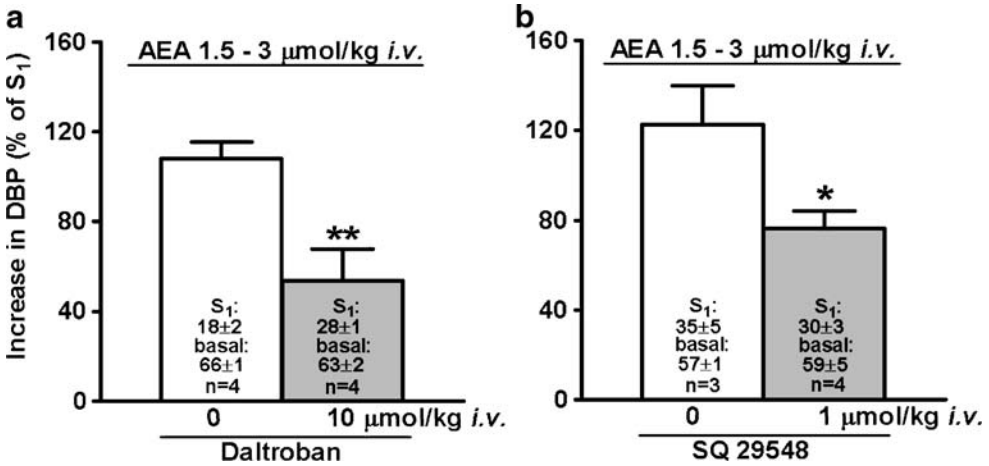

Fig. 4 Influence of intravenous (i.v.) injection of **a** daltroban and **b** SQ 29548 on the increase in diastolic blood pressure (DBP) induced by i.v. administration of anandamide (AEA) in urethane-anaesthetized rats. AEA was injected twice (S_1 and S_2, 15 min apart). Daltroban, SQ 29548 or their solvents were administered 5 min before S_2. S_1 and S_2 were calculated as percent of the respective basal values (values of S_1 and basal DBP (in millimetres of Hg) determined immediately prior to S_1 are given on *bottom of the columns*). Mean ± SEM of three to four rats. *P<0.05, **P<0.01, compared with the corresponding control

pressor effect of the endocannabinoid was reduced by furegrelate by about 60% (Fig. 6b).

Influence of MK-801, ICI 118551 and adrenalectomy on the pressor response to anandamide

As shown in Fig. 7a, the pressor response to i.c.v. anandamide 0.03 µmol per animal was diminished by the i.v. injection of the non-selective NMDA receptor antagonist MK-801 and the β_2-adrenoceptor antagonist ICI 118551 (1 µmol/kg, each) by about 70 and 90%, respectively. The pressor effect of i.c.v. anandamide (0.03 µmol per animal) in anaesthetized, bilaterally adrenalectomized rats treated with cortisol (3 µmol/kg, i.m.) was reduced by about 60%. In sham-operated animals, this response amounted to about 15% of the basal value (Fig. 7b).

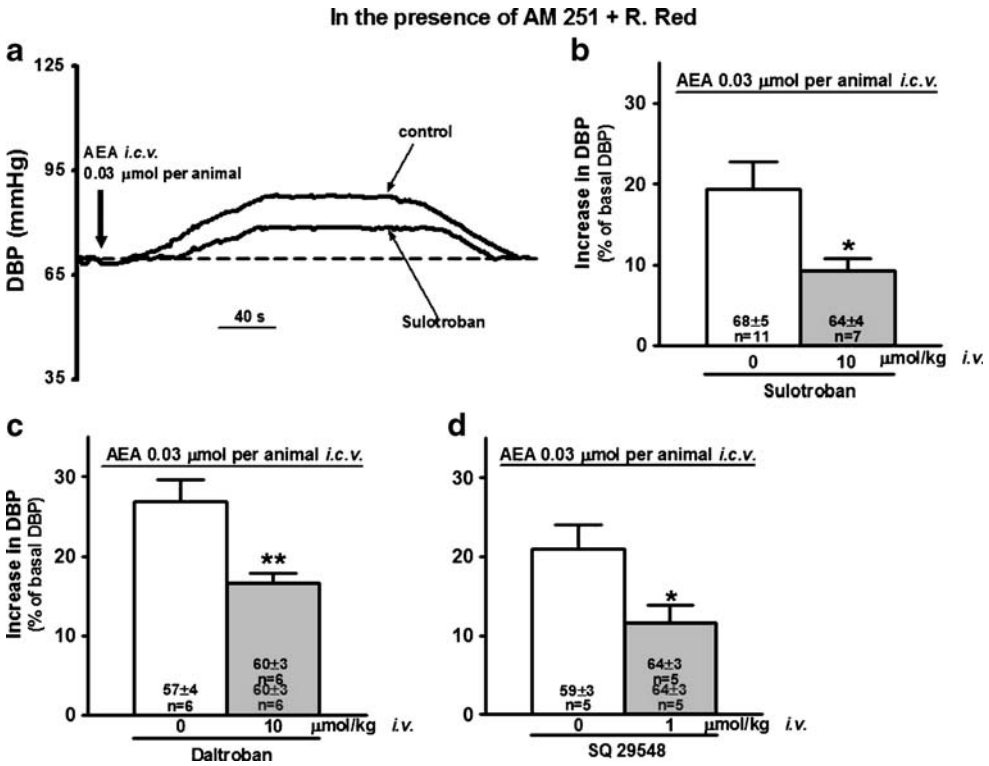

Fig. 5 Influence of intravenous (i.v.) injection of **a**, **b** sulotroban, **c** daltroban and **d** SQ 29548 on the increase in diastolic blood pressure (DBP) induced by i.c.v. administration of anandamide (AEA) in urethane-anaesthetized rats. AEA was given 5 min after combined intravenous (i.v.) injection of AM 251 plus ruthenium red (AM 251+R. Red; 3 µmol/kg, each) and of TP receptor antagonists or their solvents. Mean ± SEM of five to 11 rats. *P<0.05, **P<0.01, compared with the corresponding control. Basal DBP determined immediately before administration of AEA is given on *bottom of the columns* (in millimetres of Hg)

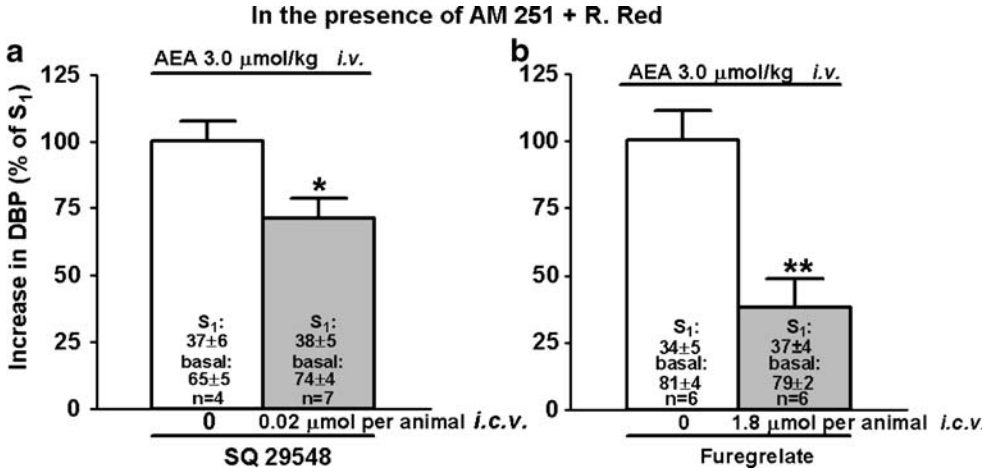

Fig. 6 Influence of i.c.v. injection of **a** SQ 29548 and **b** furegrelate on the increase in diastolic blood pressure (DBP) induced by intravenous (i.v.) administration of anandamide (AEA) in urethane-anaesthetized rats. AEA was injected twice (S_1 and S_2). AM 251 plus ruthenium red (AM 251+R. Red; 3 μmol/kg, each) were injected i.v. 5 min before S_1. SQ 29548 or its solvent was injected 10 min after S_1 and 5 min before S_2. Furegrelate or its solvent was given 10 min after S_1 and 30 min before S_2. S_1 and S_2 were calculated as % of the respective basal values (values of S_1 and basal DBP (in millimetres of Hg) determined immediately prior to S_1 are given on *bottom of the columns*). Mean ± SEM of four to seven rats. *$P<0.05$, **$P<0.01$, compared with the corresponding control

Binding

In saturation binding experiments on washed rat platelets, using ^{3}H-SQ 29,548 at eight concentrations, a K_D value of 3.8±0.4 nM with a maximum number of binding sites (B_{max}) of 1309±48 fmol/mg or 0.37±0.01 fmol per 10^6 platelets was determined; Scatchard analysis revealed a straight line with a Hill coefficient (n_H) of unity (Fig. 8a). Unspecific binding (determined with unlabelled SQ 29,548 50 μM) was 20% of total binding for ^{3}H-SQ 29,548 3 nM. In competition binding experiments, binding of ^{3}H-SQ 29,548 3 nM was inhibited monophasically (n_H near unity) by the TP receptor agonist U-46,619 (pK_i 7.58±0.19) but not affected by anandamide and methanandamide at concentrations ranging from 0.1 to 10 μM (Fig. 8b or not shown).

Discussion

The present paper aimed at further clarifying the mechanisms underlying the brief pressor response (phase II) of the triphasic cardiovascular effect of the endocannabinoid anandamide in anaesthetized rats and especially to investigate a

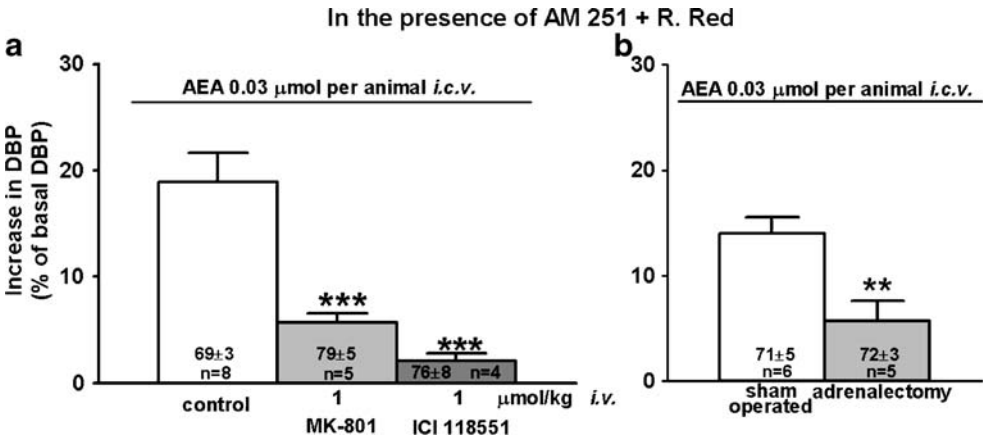

Fig. 7 Influence of **a** intravenous (i.v.) injection of MK-801 or ICI 118551 or **b** of adrenalectomy on the increase in diastolic blood pressure (DBP) induced by i.c.v. administration of anandamide (AEA) in urethane-anaesthetized rats. All experiments were performed in the presence of AM 251 plus ruthenium red (AM 251+R. Red; 3 μmol/kg, each) given i.v. 5 min before AEA. MK-801, ICI 118551 or their solvents were also injected i.v. 5 min before AEA. Adrenalectomy or sham operation was performed about 20 min before AEA. Mean ± SEM of four to eight rats. **$P<0.01$, ***$P<0.001$, compared with the corresponding control. Basal DBP determined immediately before administration of AEA is given on *bottom of the columns* (in millimetres of Hg)

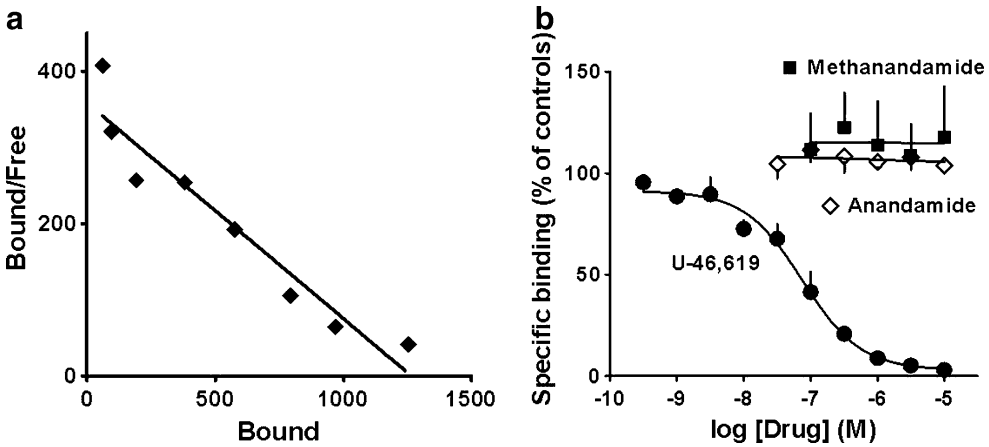

Fig. 8 Specific ^{3}H-SQ 29,548 binding to washed rat platelets. **a** Saturation binding experiments. Shown is the transformation of the data (total binding minus unspecific binding) into a Scatchard plot. **b** Effect of anandamide, methanandamide and U-46,619 on the binding of ^{3}H-SQ 29,548 3 nanomolars. Mean ± SEM of three to four experiments in triplicate

possible role of thromboxane A_2 (TP) receptors. Rats were anaesthetized with urethane since pentobarbitone was shown to reduce the anandamide-stimulated increase in blood pressure (Kwolek et al. 2005). Since vasopressor/vasodepressor effects are more marked at higher levels of blood pressure (Malinowska and Schlicker 1993), DBP was increased to 55–70 mmHg by vasopressin in animals with a lower level of this parameter (i.e. in all pithed and adrenalectomized rats and in some anaesthetized animals). This procedure has also been chosen in other papers in which cardiovascular effects of cannabinoids were examined (e.g. Wagner et al. 2001; Pfitzer et al. 2005). Impairment of our results by vasopressin is unlikely since the vasopressin V_{1a} receptor antagonist $d(CH_2)_5[Tyr(Me)^2Arg^8]$-vasopressin failed to affect the cardiovascular responses to anandamide (Kwolek et al. 2005).

We found that the pressor effect of anandamide (i.v.) in urethane-anaesthetized rats was reduced by about 35–50% by three TP receptor antagonists, sulotroban, daltroban and SQ 29548, administered i.v. The possibility that anandamide acts via a metabolite (see e.g. Yu et al. 1997) can be excluded since the increase in DBP induced by the stable analogue of this compound, methanandamide i.v., was inhibited by sulotroban to a similar degree. TP receptors are known to mediate potent vasoconstriction (e.g. Sellers and Stallone 2008). However, quite unexpectedly, the same dose of sulotroban which effectively reduced the pressor effect of anandamide and methanandamide in intact anaesthetized rats failed to modify the increase in DBP induced by anandamide in pithed animals. These results suggest that the TP receptors involved in the pressor effect of anandamide are located in the central nervous system.

To distinguish more directly between central and peripheral effects of anandamide, we compared the changes in cardiovascular parameters after its intravenous and intracerebroventricular administration. In contrast to the typical, well-known triphasic response to i.v. injection of anandamide (for references, see Introduction), its i.c.v. application caused only two small hypotensive responses. We assumed that the two hypotensive responses induced by anandamide i.c.v. might mask its pressor effect. Thus, experiments were performed in the presence of ruthenium red and AM 251, i.e. blockers of TRPV1 and cannabinoid CB_1 receptors, respectively. The two receptors are involved in the early and late vasodepressor response to this endocannabinoid, respectively (for references, see Introduction). Indeed, in the presence of antagonists of both receptors, a pure, dose-dependent pressor response to anandamide i.c.v. occurred. The maximal effect obtained for doses of 0.03–0.1 μmol per animal was about 20% of basal DBP. Importantly, in the presence of the TRPV1 and CB_1 receptor antagonist, the increase in DBP elicited by anandamide i.c.v. was about five times longer than the respective effect induced by this agonist given i.v. and in the absence of any antagonists.

The reversal of the hypotensive to a hypertensive effect was somewhat surprising for us since the data of our previous study (Kwolek et al. 2005) had suggested that anandamide acts via peripherally located TRPV1 and CB_1 receptors whereas the present results with i.c.v. administered anandamide suggest a central target. The results by Villanueva et al. (2009) suggest that centrally located CB_1 receptors are probably important in the initiation of endotoxic hypotension since i.c.v. administration of another CB_1 receptor antagonist, rimonabant, inhibits the fall in arterial blood pressure evoked by lipopolysaccharide in conscious and anaesthetized rats. In order to allow us to examine the influence of various antagonists or experimental conditions on the pure pressor effect of anandamide all further experiments with i.c.v. administration of drugs were performed in the presence of ruthenium red and AM 251.

Two additional series of experiments confirm the potential involvement of central prostanoid TP receptors in the action of the endocannabinoid. Thus, the pressor effect of anandamide 0.03 μmol per animal i.c.v. was

reduced again by i.v. application of the three TP receptor antagonists sulotroban, daltroban and SQ 29548, thus mimicking the inhibitory effect of the antagonists on the pressor effect of anandamide when given i.v. Secondly, i.c.v. injection of SQ 29548 diminished the increase in DBP elicited by i.v. administration of anandamide, i.e. the antagonism was also evident when the route of administration of the drugs was exchanged.

In view of the chemical relationship between anandamide (for review, see Pacher et al. 2008) and thromboxane A_2, both of which are derived from arachidonic acid, the question arises whether anandamide can directly activate the central TP receptors. This possibility can be excluded since, in contrast to the TP receptor agonist U-46,619, both anandamide and methanandamide failed to bind to TP receptors on washed rat platelets. Moreover, an inhibitor of thromboxane A_2 synthase furegrelate 1.8 μmol per animal i.c.v. reduced the pressor effect of anandamide i.v. suggesting that anandamide causes an increase in thromboxane A_2 synthesis in the brain.

In urethane-anaesthetized rats, i.c.v. administered *N*-methyl-D-aspartate (NMDA) has been shown to evoke the secretion of adrenaline and noradrenaline from the adrenals via a thromboxane A_2-mediated mechanism in the brain (Okada et al. 2008). In our previous paper, we found that central NMDA receptors mediate the pressor effect of anandamide since MK-801, a non-competitive antagonist at the latter receptors, reduced the increase in DBP induced by i.v. administered anandamide in intact but not in pithed rats (Kwolek et al. 2005). In the present study, we confirmed the above observation showing that MK-801 caused a strong inhibition (by about 70%) of the rise in DBP not only when anandamide was given i.v. but also when it was administered i.c.v. Moreover, the pressor effect of the endocannabinoid was strongly (by about 60%) reduced in adrenalectomized animals. In view of the failure of adrenalectomy to completely inhibit the anandamide-induced pressor effect, it is possible that noradrenaline released from the sympathetic nerve endings also contributes to this effect. The lack of any effect of adrenalectomy on the increase in DBP induced by anandamide i.v. found previously (Kwolek et al. 2005) can be explained by the difference in experimental conditions, i.e. the fact that those experiments were performed in the absence of TRPV1 and CB_1 receptor antagonists. Finally, we found here that the β_2-adrenoceptor antagonist ICI 118551 almost abolished the increase in DBP stimulated by i.c.v. administered anandamide. These results prove that β_2-adrenoceptors are involved in the pressor effect of anandamide and are compatible with our previous study in which MK-801 and the non-selective β-adrenoceptor antagonist propranolol reduced the pressor effect of i.v.-administered anandamide in intact but not in pithed rats (Kwolek et al. 2005).

The present data suggest that thromboxane A_2 (TP) receptors, NMDA receptors and β_2-adrenoceptors in the CNS play a role in the pressor effect of anandamide. Anandamide inhibits the release of many neurotransmitters (including glutamate) acting via presynaptic CB_1 receptors (Schlicker and Kathmann 2001) but, in the presence of a CB_1 receptor antagonist, anandamide augments postsynaptic responses mediated via NMDA receptors in rat brain slices (Hampson et al. 1998) and rodent hypoglossal motoneurons (Mukhtarov et al. 2005; potential positive allosteric effect). This putative dual effect of anandamide would explain why, in our hands, anandamide had a minor depressor effect in the absence of antagonists (when both mechanisms are simultaneously activated) but a marked pressor effect after administration of a CB_1 (and a TRPV1) receptor antagonist.

Our results do not provide an answer to the question for the exact site(s) of action of the endocannabinoid since drugs injected i.c.v. are likely to reach most of the brain regions involved in the regulation of cardiovascular function. One possible candidate is the rostral ventrolateral medulla (RVLM). In halothane-anaesthetized rats, anandamide i.v. causes a sharp increase in activity of neurons of the brain regions and in splanchic sympathetic nerves preceding the pressor phase, followed by a more prolonged rise during the phase of prolonged hypotension (Varga et al. 1996). The latter authors suggested that an additional action of anandamide at the sympathetic nerve terminal to decrease transmitter release might be the reason why the centrally trigged increase in sympathetic outflow does not lead to an increase in blood pressure. Another candidate is the nucleus paraventricularis (PVN) of the hypothalamus, in which both NMDA receptors (Herman et al. 2000) and β_2-adrenoceptors (Rainbow et al. 1984) are present. Moreover, perfusion of the PVN with NMDA increases the level of thromboxane B_2 (the inactive metabolite of thromboxane A_2) in this brain region, plasma levels of catecholamines (Okada et al. 2000) and blood pressure (Li and Pan 2007). Finally, injection of a TXA_2 mimetic into the PVN predominantly increases plasma adrenaline levels (Murakami et al. 2002).

In conclusion, the present study, which includes experiments with i.c.v. administered anandamide, confirms that central NMDA and β_2-adrenergic receptors are involved in the vasopressor response to this endocannabinoid in the anaesthetized rat. The study shows, for the first time, that receptors for thromboxane A_2 (TP receptors) play a role as well. These receptors are located in the central nervous system and are not directly activated by anandamide or by a metabolite formed from it. Anandamide rather leads to the increased formation of thromboxane A_2 or a related compound via an unknown mechanism. One point of concern is that i.c.v. administered anandamide increases blood pressure only if CB_1 (and TRPV1) receptors are

blocked simultaneously. This does, however, not argue against the possibility that i.v. administered anandamide increases blood pressure via a central site of action. The compound may be distributed to a central site where its CB_1 receptor-independent hypertensive effect overrides its CB_1 receptor-mediated hypotensive effect. The exact mechanism of the CB_1 receptor-independent effect of anandamide is unclear but may involve a positive allosteric effect on NMDA receptors. Importantly, the pressor effect of anandamide has been demonstrated to be enhanced in conscious normotensive rats (Lake et al. 1997; Gardiner et al. 2009).

Acknowledgements This work was supported by the Medical University of Białystok (Poland; grant No. 3-13579F; 3-13581F). B. Malinowska and E. Schlicker were granted by the Foundation for Polish Science (FNP; Warsaw, Poland) and the German Research Foundation (DFG; Bonn, Germany) with the Copernicus Award. M. Göthert was granted by the Foundation for Polish Science with the Alexander von Humboldt Polish Honorary Research Fellowship. The authors are indebted to the Alexander von Humboldt-Stiftung (Bonn, Germany) for generously providing some of the equipment.

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