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The endocannabinoid anandamide inhibits kinin B_1 receptor sensitization through cannabinoid CB_1 receptor stimulation in human umbilical vein

Facundo Germán Pelorosso [1], Juan Ezequiel Gago, Giannina del Rey, Sofía Diana Menéndez, Andrea Emilse Errasti [2], Rodolfo Pedro Rothlin *

Departamento de Farmacología, Facultad de Medicina, Universidad de Buenos Aires, 2155 Paraguay St., 9th floor, Ciudad Autónoma de Buenos Aires (CP 1121), Argentina

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ABSTRACT

The possible inhibition of kinin B_1 receptor up-regulation by arachidonoylethanolamide (anandamide) was evaluated in isolated human umbilical vein. Anandamide and its metabolically stable analogue, R-N-(2-Hydroxy-1-methylethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (R-(+)-methanandamide), produced a selective and dose-dependent inhibition of kinin B_1 receptor-sensitized contractile responses. The inhibitory effect of anandamide on B_1 receptor-sensitized responses failed to be modified either by 5-biphenyl-4-ylmethyl-tetrazole-1-carboxylic acid dimethylamide (LY2183240), a selective anandamide uptake inhibitor, or 6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-y l](4-methoxyphenyl) methanone (AM630), selective cannabinoid CB_2 receptor antagonist. However, the cannabinoid CB_1 receptor antagonist, N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophen yl)-4-methyl-1H-pyrazole-3-carboxamide (AM251), abolished anandamide effects on kinin B_1 receptor sensitization. The present results provide strong pharmacological evidence indicating that endocannabinoid anandamide inhibits kinin B_1 receptor up-regulation through cannabinoid CB_1 receptor stimulation in human umbilical vein.

1. Introduction

The actions of kinins are mediated through the stimulation of two subtypes of G-protein-coupled receptors, B_1 and B_2. Whereas kinin B_2 receptor expression is constitutive and mediates most of the *in vivo* effects of kinins, B_1 receptors are hardly expressed in nontraumatized tissues (reviewed in Leeb-Lundberg et al., 2005). However, they can be up-regulated under certain conditions, such as those after tissue injury and infection (Regoli et al., 1977; Sardi et al., 1997) and by a wide array of nuclear factor kappa-B (NF-κB) activating agents (Sardi et al., 2000). Evidence from knock-out mice has revealed that kinin B_1 receptor is critically required for a number of important physiological and pathophysiological functions *in vivo*, including blood pressure homeostasis, inflammation, and nociception (Pesquero et al., 2000). Interestingly, it has been demonstrated that gene deletion or pharmacological inhibition of the B_1 receptor in mice practically abolishes the development and maintenance of neuropathic pain (Ferreira et al., 2005; Gabra and Sirois, 2002), a condition accompanied by B_1 receptor up-regulation in tissues important for the detection, transmission and modulation of pain such as dorsal root ganglia and spinal cord (Ferreira et al., 2005).

At the same time, recent animal studies indicate that several cannabinoid ligands are very effective against pain of neuropathic origin (Bridges et al., 2001; Costa et al., 2004; Guindon and Beaulieu, 2006; Herzberg et al., 1997). Antinociceptive and anti-inflammatory effects of cannabinoids are complex and can be mediated by several mechanisms. These include actions at several anatomical loci, including central nervous system, spinal cord, dorsal root ganglia and peripheral nerves, and may involve modulation of cytokine and chemokine production, expression of adhesion molecules, and the migration, proliferation, and apoptosis of inflammatory cells (reviewed in Pacher et al., 2006). Interestingly, it has recently been shown that anandamide may inhibit NF-κB activation in various cell types (Nakajima et al., 2006; Sancho et al., 2003). These findings raise the question of whether endocannabinoids might exert some of their actions (e.g. antinociception in neuropathic pain) through inhibition of the kinin B_1 receptor. By employing an *in vitro* human umbilical vein contractility assay, we provide here strong pharmacological evidence indicating that endocannabinoid anandamide inhibits human kinin B_1 receptor up-regulation.

* Corresponding author. Tel./fax: +54 011 4962 0300.
E-mail address: farmaco3@fmed.uba.ar (R.P. Rothlin).
[1] Facundo Germán Pelorosso is a research fellow of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).
[2] Andrea Emilse Errasti is member of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

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2. Materials and methods

2.1. Contractility studies

Human umbilical cords were obtained from normal full term deliveries and excised midway between the child and the placenta. Approval from a local ethics committee and written informed consent were obtained. Isolation of human umbilical vein rings and measurement of changes in isometric tension were carried out as described previously (Sardi et al., 1997). Briefly, immediately after dissection, human umbilical vein rings were suspended in 10 ml organ baths and stretched with an initial tension of 3 to 5 g. It has been previously shown that tissue sensitivity to B_1 receptor agonists increases gradually in human umbilical vein as a function of *in vitro* incubation time (Sardi et al., 1997). Therefore, tissues were allowed to stabilize for 150 min before contractile cumulative concentration–response curves were obtained for either Lys-des-Arg^9-bradykinin (Lys-des-Arg^9-BK; the natural kinin B_1 receptor agonist), Sar^0-[D-Phe^8]des-Arg^9-bradykinin (Sar^0-[D-Phe^8]des-Arg^9-BK; a metabolically stable synthetic B_1 receptor agonist) or 5-hydroxytryptamine (5-HT; an unrelated contractile agent). Only one agonist concentration–response curve was performed on a single ring.

Anandamide and R-(+)-methanandamide effects on kinin B_1 receptor up-regulation were evaluated by exposure of human umbilical vein rings to several concentrations of those cannabinoids during the complete stabilization period prior to the construction of the concentration–response curves to the kinin agonists. When 5-biphenyl-4-ylmethyl-tetrazole-1-carboxylic acid dimethylamide (LY2183240, anandamide uptake inhibitor), N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophen yl)-4-methyl-1H-pyrazole-3-carboxamide (AM251, cannabinoid CB_1 receptor antagonist) and 6-Iodo-2-methyl-1-[2-(4-morpholinyl) ethyl]-1H-indol-3-y l](4-methoxyphenyl)methanone (AM630, cannabinoid CB_2 receptor antagonist) were used, they were added to the bath 15 min before and throughout the incubation with anandamide. None of the treatments applied before the construction of the concentration–response curves produced direct modifications in human umbilical vein tone. All experiments were carried out in parallel in rings from the same umbilical vein. At the end of each concentration–response curve, 10 μM 5-HT was applied to determine the tissue maximal contractile response (Altura et al., 1972).

2.2. Data analysis and statistical procedures

All data are expressed as mean ± S.E.M. The number of experiments *n* represents the number of rings from different cords tested. Contractile responses are expressed as percentage of tissue maximum response elicited by 10 μM 5-HT. The estimates of EC_{50} values (i.e., the agonist concentration that produces 50% of the maximal response), the maximal response (E_{max}) and n_H were obtained using ALLFIT (DeLean et al., 1978).

Statistical analysis was performed by means of unpaired Student's *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett or Tukey's post-test, when appropriate. *P* values lower than 0.05 were taken to indicate significant differences.

2.3. Drugs, chemicals reagents and other materials

Anandamide, R-(+)-methanandamide, LY2183240, AM630 and AM251 were purchased from Tocris Bioscience (Ellisville, MO); 5-HT creatine sulfate complex from Sigma/RBI (Natick, MA); Lys-des-Arg^9-BK from Bachem Biosciences Inc. (King of Prussia, PA); Sar^0-[D-Phe^8]des-Arg^9-BK from Phoenix Pharmaceuticals Inc. (Belmont, CA).

Preparation of all stock peptide solutions and their subsequent dilutions were performed in glass bidistilled water. Stock solutions were stored in frozen aliquots and thawed and diluted daily. Stock cannabinoid solutions in absolute ethanol were stored at −20 °C, protected from light and aliquoted for daily use. The final concentrations of ethanol in the bath solutions were no higher than 0.25% v/v. Preliminary experiments showed that 0.25% v/v ethanol fails to modify Sar^0-[D-Phe^8]des-Arg^9-BK induced responses in human umbilical vein (data not shown). Nevertheless, all control trials were performed in the presence of the corresponding concentration of ethanol.

3. Results

3.1. Anandamide inhibits kinin B_1 receptor sensitization in human umbilical vein

While no significant effects were observed at 1 and 5 μM, continuous exposure to 10 μM anandamide inhibited Sar^0-[D-Phe^8]des-Arg^9-BK-induced responses in human umbilical vein after 150 min of *in vitro* incubation (Fig. 1A; Table 1). Accordingly, 10 μM anandamide inhibited Lys-des-Arg^9-BK-induced responses in human umbilical vein when applied in the same fashion (Table 1). On the other hand, short exposure (15 min before and throughout the construction of the concentration–response curve) to 10 μM anandamide failed to modify kinin B_1 receptor mediated responses induced by either agonist (Table 1). In addition, neither pEC_{50} nor maximal response to 5-HT, an unrelated agonist, were modified by continuous exposure to 10 μM anandamide (data not shown).

3.2. R-(+)-methanandamide inhibits kinin B_1 receptor sensitization in human umbilical vein

The stable anandamide analogue, R-(+)-methanandamide (1, 5 and 10 μM) produced a dose dependent inhibition of Sar^0-[D-Phe^8]

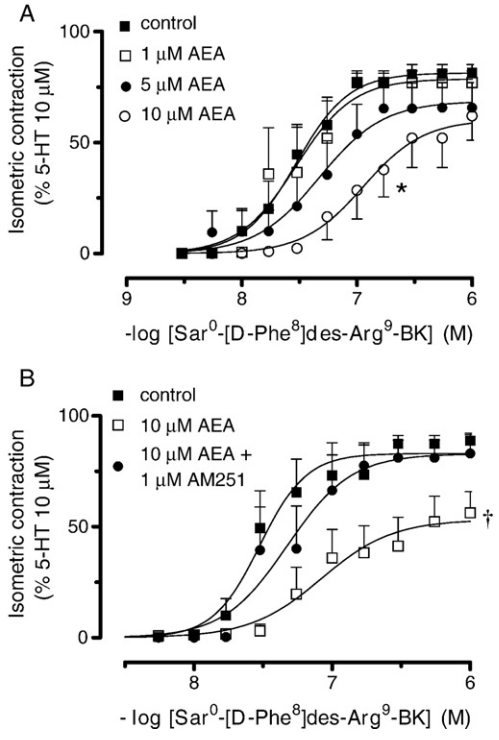

Fig. 1. A. Effects of continuous exposure to anandamide on Sar^0-[D-Phe^8]des-Arg^9-BK-elicited responses in isolated human umbilical vein. All data represent the mean of 5 to 8 independent experiments (error bars indicate S.E.M.). B. Effects of CB_1 receptor antagonist, AM251, on endocannabinoid effects on Sar^0-[D-Phe^8]des-Arg^9-BK-elicited responses in isolated human umbilical vein. All data represent the mean of 7 independent experiments (error bars indicate S.E.M.). * Represents significant differences in pEC_{50} vs. control ($P<0.05$). † represents significant differences in Emax vs. control ($P<0.05$).

Table 1
Effects of anandamide on kinin B_1 receptor sensitization in human umbilical vein

	pEC_{50}	E_{max}	n
	mean ± S.E.M.	mean ± S.E.M. (% 5-HT 10 μM)	
Concentration–response curve to Sar^0-[D-Phe^8]des-Arg^9-BK			
Control	7.53 ± 0.07	81.5 ± 4.2	8
Anandamide continuous treatment (150 min)			
1 μM	7.53 ± 0.10	78.8 ± 5.9	5
5 μM	7.33 ± 0.14	68.7 ± 7.8	7
10 μM	6.95 ± 0.14[a]	60.4 ± 9.0	7
Control	7.61 ± 0.04	80.6 ± 3.0	6
Anandamide short treatment (15 min)			
10 μM	7.70 ± 0.14	72.1 ± 6.9	6
Control	7.79 ± 0.06	78.2 ± 3.4	6
R-(+)-methanandamide continuous treatment			
1 μM	7.55 ± 0.04[a]	77.9 ± 3.1	6
5 μM	7.33 ± 0.06[a]	75.8 ± 4.2	6
10 μM	7.32 ± 0.05[a]	65.2 ± 2.3[a]	6
Control	7.47 ± 0.22	81.3 ± 6.4	5
R-(+)-methanandamide 10 μM short treatment	7.54 ± 0.09	75.9 ± 5.2	5
Control	7.80 ± 0.06	73.2 ± 3.6	7
Anandamide 10 μM continuous treatment	7.44 ± 0.07[a]	58.6 ± 3.8	7
Anandamide plus LY2183240 10 nM	7.31 ± 0.14[a]	50.7 ± 5.6[a]	7
LY2183240 10 nM	7.69 ± 0.08	75.1 ± 4.3	7
Control	7.53 ± 0.06	83.1 ± 4.2	7
Anandamide 10 μM continuous treatment	7.08 ± 0.13[a]	53.3 ± 6.7[a]	7
Anandamide plus AM251 1 μM	7.33 ± 0.09	82.9 ± 6.1	7
AM251 1 μM	7.26 ± 0.09	77.9 ± 6.5	7
Control	7.75 ± 0.08	81.4 ± 4.4	6
Anandamide 10 μM continuous treatment	7.31 ± 0.05[a]	81.1 ± 3.8	6
Anandamide plus AM630 1 μM	7.35 ± 0.05[a]	62.9 ± 3.1[a]	6
AM630 1 μM	7.68 ± 0.05	77.6 ± 3.2	6
Concentration–response curve to Lys-des-Arg^9-BK			
Control	8.61 ± 0.07	77.3 ± 3.5	6
Anandamide 10 μM continuous treatment	7.79 ± 0.08[a]	77.0 ± 4.1	6
Control	8.48 ± 0.10	70.1 ± 4.3	6
Anandamide 10 μM short treatment	8.38 ± 0.07	77.4 ± 3.0	6

[a] Represents significant differences vs. control values ($P<0.05$).

des-Arg^9-BK-induced responses in isolated human umbilical vein when applied continuously (Table 1). Conversely, short exposure to 10 μM R-(+)-methanandamide failed to modify kinin B_1 responses in this tissue (Table 1). Likewise, continuous treatment with this anandamide analogue was unable to alter 5-HT-elicited responses in human umbilical vein (data not shown).

3.3. Lack of effect of endocannabinoid uptake inhibitor, LY2183240, on anandamide inhibition of kinin B_1 receptor sensitization in human umbilical vein

10 nM LY2183240, a selective anandamide uptake inhibitor, failed to modify endocannabinoid inhibition of kinin B_1 receptor mediated responses in human umbilical vein (Table 1). In addition, LY2183240 alone did not change Sar^0-[D-Phe^8]des-Arg^9-BK-induced responses in isolated human umbilical vein (Table 1).

3.4. Reversal of anandamide inhibition of kinin B_1 receptor sensitization by cannabinoid CB_1 receptor antagonist/inverse agonist AM251 in human umbilical vein

1 μM AM251, selective cannabinoid CB_1 receptor antagonist/inverse agonist, produced the complete reversal of anandamide inhibition of kinin B_1 receptor sensitization in human umbilical vein (Table 1; Fig. 1B). Moreover, AM251 alone failed to modify responses to the B_1 receptor agonist (Table 1).

3.5. Lack of effect of cannabinoid CB_2 receptor antagonist/inverse agonist, AM630, on anandamide inhibition of kinin B_1 receptor sensitization in human umbilical vein

1 μM AM630, selective cannabinoid CB_2 receptor antagonist/inverse agonist, did not reverse anandamide inhibitory effect on kinin B_1 receptor sensitization in human umbilical vein (Table 1). Also, AM630 lacked any effects on Sar^0-[D-Phe^8]des-Arg^9-BK-induced responses *per se* (Table 1).

4. Discussion

Sensitization to kinin B_1 receptor agonists in isolated human umbilical vein is a *bona fide* system to study kinin B_1 receptor up-regulation in a human tissue (Sardi et al., 2000). In this model, B_1 receptor-mediated contractile responses develop from an initial null level in freshly isolated tissues and increase in magnitude as a function of *in vitro* incubation time (Sardi et al., 1997). This sensitization process is dependent on *de novo* synthesis of receptors since it is abolished by transcription, translation, protein trafficking and glycosylation inhibitors (Sardi et al., 1998; Sardi et al., 1999). It has also been shown in this tissue that pro and anti-inflammatory mediators modulate B_1 receptor sensitization (Sardi et al., 1998; Sardi et al., 1999; Sardi et al., 2002), and that NF-κB activation plays a key role in the development of B_1 receptor-sensitized responses (Sardi et al., 1999; Sardi et al., 2002). Interestingly, it has recently been shown that anandamide may inhibit NF-κB activation in various cell types (Nakajima et al., 2006; Sancho et al., 2003).

In the present work we show that human umbilical vein rings continuously exposed to anandamide develop a dose dependent inhibition of vasoconstrictor responses elicited by Sar^0-[D-Phe^8]des-Arg^9-BK, a metabolically stable synthetic kinin B_1 receptor agonist. In addition, the inhibitory effect of anandamide was reproduced when using the endogenous B_1 receptor agonist, Lys-des-Arg^9-BK. Further analysis revealed that anandamide effect is time dependent since a short exposure to the endocannabinoid (only 15 min before the construction of the concentration–response curve to the kinin B_1 receptor agonists) failed to modify such responses. A wide array of inhibitors of kinin B_1 receptor up-regulation have been shown to be ineffective when applied to tissues only minutes before the agonist stimulation (Sardi et al., 1998; Sardi et al., 1999). In agreement with this, the present results rule out a direct acute effect of anandamide on vascular tone in general or in kinin B_1 receptor signal transduction in particular, in human umbilical vein. Moreover, contractile responses induced by 5-HT, an unrelated agonist, were without modification by continuous exposure to the endocannabinoid. Taken as a whole these results support our hypothesis that anandamide inhibits kinin B_1 receptor up-regulation in isolated human umbilical vein.

In several tissues, actions of anandamide are usually terminated by its rapid uptake by intact cells by the putative anandamide membrane transporter and subsequent hydrolysis by the enzyme fatty acid amide hydrolase (FAAH; reviewed in Pacher et al., 2006). However, anandamide metabolites derived from FAAH activity, namely arachidonic acid and ethanolamine can sometimes mediate its actions. Accordingly, it has been demonstrated in some models that, whereas anandamide displays biological activity, its metabolically stable analogue and CB receptor agonist, R-(+)-methanandamide, lacks such activity when evaluated under the same conditions (Wahn et al., 2005). To evaluate if anandamide activity on kinin B_1 receptor sensitization in human umbilical vein is related to the generation of its hydrolytic products, we performed experiments employing R-(+)-methanandamide. In our model, this anandamide analogue retained its inhibitory activity on Sar^0-[D-Phe^8]des-Arg^9-BK-elicited responses. Therefore, our results obtained with R-(+)-methanandamide strongly suggest that anandamide does not mediate the inhibition of kinin B_1 receptor sensitization in human umbilical vein via either one of its metabolites.

In addition, anandamide has been shown to activate the vanilloid receptor 1 ($TRPV_1$) at an intracellular site. In agreement with this, the actions of exogenous anandamide at $TRPV_1$ can be blocked by selective inhibitors of endocannabinoid uptake (De Petrocellis et al., 2001). Here we show that LY2183240, a novel potent selective anandamide uptake inhibitor (Moore et al., 2005), failed to modify anandamide inhibition of kinin B_1 receptor sensitization in human umbilical vein, suggesting that in this model the endocannabinoid acts at an extracellular site.

Mammalian tissues express at least two types of cannabinoid receptor, CB_1 and CB_2, both G protein coupled (reviewed in Howlett et al., 2002). Although cannabinoid CB_1 receptors are found predominantly at central and peripheral nerve terminals where they mediate inhibition of transmitter release, they have also been found in various tissues including the heart and vasculature (Bonz et al., 2003; Gebremedhin et al., 1999; Liu et al., 2000). On the other hand, cannabinoid CB_2 receptors occur mainly on immune cells, one of their roles being to modulate cytokine release (Howlett et al., 2002). anandamide is a partial or full agonist of cannabinoid CB_1 receptors, depending on the tissue and biological response measured. Although it also binds cannabinoid CB_2 receptors, it has very low efficacy and may act as an antagonist (Pacher et al., 2006). To evaluate the role of cannabinoid CB_1 receptors on anandamide inhibition of kinin B_1 receptor sensitization in human umbilical vein we performed experiments in the presence of AM251. This ligand is a potent and selective cannabinoid CB_1 receptor antagonist/inverse agonist with a reported K_i value of 7.49 nM at cannabinoid CB_1 receptors and a 306-fold selectivity over cannabinoid CB_2 receptors (K_i 2.29 μM) (Lan et al., 1999). In our model, 1 μM AM251 produced a complete reversal of anandamide inhibition of kinin B_1 receptor-mediated responses. This result constitutes strong pharmacological evidence for the involvement of cannabinoid CB_1 receptor activation in anandamide-induced inhibition of B_1 receptor sensitization in isolated human umbilical vein.

Further experiments carried out to evaluate the role of cannabinoid CB_2 receptor subtype in anandamide effects were performed with AM630, a cannabinoid CB2 antagonist/inverse agonist (K_i = 31.2 nM) 165-fold selective over cannabinoid CB_1 receptors (K_i 5.1 μM) (Ross et al., 1999). Exposure of human umbilical vein rings to 1 μM AM630 failed to modify anandamide effects on kinin B_1 induced-responses. This result suggests that cannabinoid CB_2 receptor is not involved in the inhibitory action of anandamide on kinin B_1 receptor sensitization in human umbilical vein.

In summary, using an *in vitro* human model, the present work provides novel pharmacological evidence indicating that endocannabinoid anandamide, through cannabinoid CB_1 receptor stimulation, inhibits kinin B_1 receptor sensitization, possibly by inhibition of its up-regulation. This finding may be relevant for the understanding of endocannabinoid mechanisms of action in inflammatory and painful processes and for the rational development of new pharmacotherapeutic approaches.

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