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The anandamide transport inhibitor AM404 activates vanilloid receptors

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Abstract

The possibility that the anandamide transport inhibitor *N*-(4-hydroxyphenyl)-5,8,11,14-eicosatetraenamide (AM404), structurally similar to the vanilloid receptor agonists anandamide and capsaicin, may also activate vanilloid receptors and cause vasodilation was examined. AM404 evoked concentration-dependent relaxations in segments of rat isolated hepatic artery contracted with phenylephrine. Relaxations were abolished in preparations pre-treated with capsaicin. The calcitonin-gene related peptide (CGRP) receptor antagonist CGRP-(8-37) also abolished relaxations. The vanilloid receptor antagonist capsazepine inhibited vasodilation by AM404 and blocked AM404-induced currents in patch-clamp experiments on *Xenopus* oocytes expressing the vanilloid subtype 1 receptor (VR1). In conclusion, AM404 activates native and cloned vanilloid receptors.

Keywords: Cannabinoid; Capsaicin; Neuron; Afferent; Pain; Receptor; Sensory; Vasodilation

1. Introduction

The anandamide transport inhibitor *N*-(4-hydroxyphenyl)-5,8,11,14-eicosatetraenamide (AM404) has been developed to prevent inactivation of anandamide by a cellular re-uptake mechanism and thereby prolong the biological effects of anandamide (Beltramo et al., 1997). This mechanism of inactivation of anandamide has been suggested to be of importance, e.g., in blood pressure regulation, since AM404 potentiated hypotensive responses to anandamide in guinea-pigs (Calignano et al., 1997). Initially, we attempted to investigate the influence of the anandamide transporter on the vasodilator action of anandamide in rat isolated hepatic artery. Anandamide relaxes this blood vessel via activation of vanilloid receptors present on perivascular sensory nerves and the subsequent release of vasodilator peptides such as calcitonin-gene related peptide (CGRP) (Zygmunt et al., 1999). However, we found that pre-treatment of rat hepatic arteries with AM404 did not potentiate the vasodilation induced by anandamide, but rather inhibited this response. The unsaturated fatty acyl chain combined with a vanillyl-like moiety makes AM404 structurally similar to both anandamide and capsaicin (Fig. 1). We therefore explored the possibility that this compound acts as a vanilloid receptor agonist causing receptor desensitization and/or depletion of sensory neruopeptides. This could be the reason why prior treatment of hepatic arteries with AM404 inhibited rather than potentiated the vasodilator effect of anandamide.

2. Materials and methods

2.1. Recording of tension

Experiments were performed on hepatic arteries from female Wistar–Hannover rats (200–250 g) as described (Zygmunt et al., 1998). Briefly, the arteries were cut into ring segments and mounted in tissue baths, containing aerated physiological salt solution (5% CO_2 and 95% O_2, 37°C, pH 7.4). Relaxant responses were studied in prepara-

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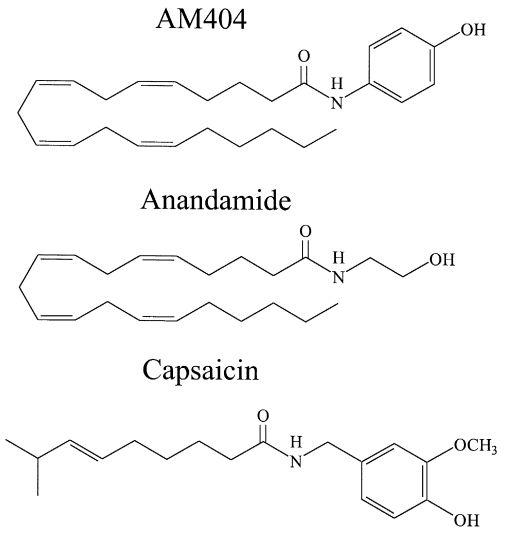

Fig. 1. AM404 is structurally related to anandamide and capsaicin. The 2-hydroxyethyl group in anandamide is substituted with a 4-hydroxyphenyl group in AM404.

tions contracted with phenylephrine. When stable contractions were obtained, AM404 was added cumulatively to determine concentration–response relationships. The incubation time with CGRP-(8-37), capsazepine, and *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride (SR-141716A) was 30 min. Some preparations were pre-treated with capsaicin for 1 h followed by washout for 20 min. Each vessel segment was exposed to only one treatment. All experiments were performed in the presence of *N*ω-nitro-L-arginine (0.3 mM) and indomethacin (10 μM) to eliminate any contribution of nitric oxide and cyclooxygenase products, respectively (Zygmunt et al., 1998). The experiments have been carried out with the approval of the local ethics committee.

2.2. Patch-clamp

Xenopus oocytes were prepared as described (Caterina et al., 1997) and injected with 10 ng of vanilloid subtype 1 receptor (VR1) cRNA. Currents were recorded in excised inside-out membrane patches of oocytes as described (Caterina et al., 1997). The membrane was held at −40 mV. Both the pipette and bath solution contained (in mM): 10 Tris, 150 CsCl, 1 $MgCl_2$, 1 EGTA, pH 7.4. Membrane patches were first briefly exposed to a threshold concentration of capsaicin to check for functional VR1 responses and then to AM404. Capsazepine was added when the AM404-induced current had reached its maximum. All experiments were performed at room temperature. Drugs were prepared as ethanol stocks and diluted with bath solution sonicated for 5 min in ice water prior to use.

2.3. Calculations and statistics

Relaxations are expressed as percentage reversal of the phenylephrine-induced contraction (100% indicates a complete relaxation). The drug concentrations eliciting 50% (EC_{50}) and maximal relaxation (E_{max}) were calculated as described (Zygmunt et al., 1998). Data are presented as mean ± S.E.M. (vertical lines in figures), and *n* indicates the number of vascular segments (animals) or cells examined. Statistical analysis was performed by Student's *t*-test (two-tailed) and statistical significance was accepted when $P < 0.05$.

2.4. Drugs

AM404, capsaicin, capsazepine (Tocris); SR141716A (Sanofi Winthrop) were all dissolved in ethanol. Distilled water was used as solvent for L-phenylephrine hydrochloride, *N*ω-nitro-L-arginine, human CGRP-(8-37) (Sigma); indomethacin (Confortid®, Dumex).

3. Results

3.1. Effect of capsaicin and CGRP-(8-37) on AM404-induced vasodilation

AM404 induced concentration-dependent relaxations in hepatic arteries of the rat (Fig. 2A,B). The pEC_{50} and E_{max} values were 7.4 ± 0.1% and 97 ± 2%, respectively (n = 10). Pre-treatment of preparations with capsaicin (10 μM) abolished AM404-induced relaxations (Fig. 2A). Likewise, the CGRP receptor antagonist CGRP-(8-37) at 3 μM abolished relaxations induced by AM404 (Fig. 2A).

3.2. Effect of capsazepine and SR141716A on AM404-induced vasodilation

The vanilloid receptor antagonist capsazepine (3 μM) caused a significant right-ward shift of the concentration–response curve for AM404 (Fig. 2B). The pEC_{50} values were 7.3 ± 0.2 and 6.4 ± 0.2 in the absence and presence of capsazepine, respectively (n = 5–7). In contrast, the cannabinoid CB_1 receptor antagonist SR141716A (0.3 μM) was without effect on AM404-induced relaxations (Fig. 2B).

3.3. Effect of AM404 on VR1 currents

AM404 at a concentration of 3 μM evoked typical ion currents in excised inside-out membrane patches of *Xenopus* oocytes expressing VR1 (n = 4, Fig. 2C). The magni-

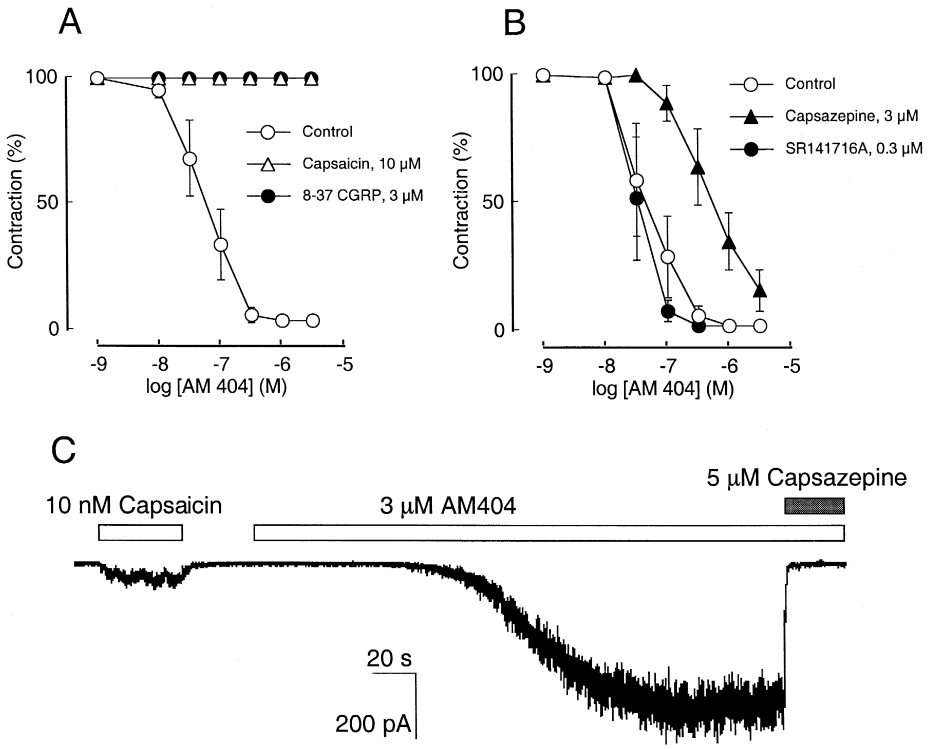

Fig. 2. Concentration-dependent relaxations induced by AM404 (A) in the absence (control) and presence of the CGRP receptor antagonist CGRP-(8-37), and in preparations pre-treated with capsaicin as well as (B) in the absence (control) and presence of the vanilloid receptor antagonist capsazepine or the CB_1 receptor antagonist SR141716A. Arteries were contracted by phenylephrine in the presence of *N*-ω-nitro-L-arginine (0.3 mM) and indomethacin (10 μM). Data are presented as mean ± S.E.M. ($n = 5–7$). (C) Representative trace of four separate experiments showing AM404-induced current and its susceptibility to capsazepine in *Xenopus* oocytes expressing VR1. A threshold concentration of capsaicin was used to confirm a functional VR1 response without causing desensitization and thereby affecting the response to the subsequent application of AM404.

tude of these currents were 3–10-fold greater than those evoked by a threshold concentration (10 nM) of capsaicin, which was used to verify a functional VR1 response. Higher concentrations of capsaicin were not used to avoid desensitization of VR1, which otherwise could affect the response to subsequent application of AM404. Capsazepine (5 μM) completely reversed AM404-induced currents ($n = 4$, Fig. 2C). Uninjected oocytes (control) did not respond to AM404 ($n = 2$; data not shown).

4. Discussion

The present study shows that AM404 is a vasodilator and activator of VR1 expressed in *Xenopus* oocytes. Pretreatment of rat hepatic arteries with capsaicin abolished AM404-induced relaxations indicating that sensory nerves are involved in the vasodilator action of AM404. The fact that the CGRP receptor antagonist CGRP-(8-37) abolished vasodilation induced by AM404 suggests that CGRP is the mediator of such relaxations. Indeed, anandamide, which releases CGRP from sensory nerves in the rat hepatic artery, is unable to cause vasodilation when the CGRP receptor is blocked by CGRP-(8-37) (Zygmunt et al., 1999). In the present study, the relaxant effect of AM404 is inhibited by the vanilloid receptor antagonist capsazepine (Bevan et al., 1992), which competitively inhibits relaxations evoked by capsaicin and methanandamide in the rat hepatic artery (Zygmunt et al., 1999). The right-ward shift of the concentration–response curve to AM404 caused by 3 μM capsazepine is of the same magnitude as when capsaicin is used as an agonist (Zygmunt et al., 1999) indicating that AM404 is acting on vanilloid receptors. Capsazepine also blocks currents through VR1 induced by capsaicin and andandamide (Caterina et al., 1997; Zygmunt et al., 1999). We found in the present study that 3 μM AM404, a concentration which caused complete vasodilation, evoked VR1 currents of similar amplitudes as other vanillyl-amides with long fatty acyl chains including olvanil (Melck et al., 1999). Interestingly, AM404 and these compounds induce VR1 currents with a much slower action compared to capsaicin and anandamide (Caterina et al., 1997; Melck et al., 1999; Zygmunt et al., 1999). As expected, capsazepine blocked AM404-induced currents in the present study.

One could argue that AM404 indirectly causes vasodilation by inhibiting the anandamide transporter leading to increased levels of endogenous anandamide and vanilloid receptor activation. However, the vasodilator action of AM404 in the rat hepatic artery occurs at lower concentra-

tions than those shown to inhibit the anandamide transporter (Beltramo et al., 1997; Beltramo and Piomelli, 1999; Di Marzo et al., 1998). An action of AM404 on cannabinoid CB_1 receptors seems unlikely since SR141716A, a selective CB_1 receptor antagonist when used at submicromolar concentrations (Pertwee, 1997, 1999; Zygmunt et al., 2000), was without effect on relaxations elicited by AM404. This is in agreement with findings showing that AM404, in contrast to cannabinoid CB_1 receptor agonists, does not inhibit forskolin-induced cyclic AMP accumulation (Beltramo et al., 1997). Thus, the phenolic moiety of AM404 apparently decreases the affinity for cannabinoid CB_1 receptors — K_i values being 78 nM for anandamide and 1760 nM for AM404 (Khanolkar et al., 1996). The affinity of AM404 to the cannabinoid CB_2 receptor is even less (Khanolkar et al., 1996). Interestingly, the phenolic moiety may improve the interaction with the vanilloid receptor, since AM404 is almost 10 times more potent as a vasodilator compared to anandamide in the rat hepatic artery (Zygmunt et al., 1999). In conclusion, our results show that AM404 resembles capsaicin and anandamide in our test systems and thus can be regarded as a vanilloid receptor ligand. In light of this, it is not surprising that our initial experiments showed that pre-treatment of rat hepatic arteries with AM404 prevented anandamide-induced vasorelaxation. This feature of AM404 may complicate its use as a pharmacological tool to evaluate the role of the anandamide transporter in test models containing vanilloid receptors.

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