A Role for N-Arachidonylethanolamine (Anandamide) as the Mediator of Sensory Nerve-Dependent Ca\(^{2+}\)-Induced Relaxation\(^1\)

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ABSTRACT

We tested the hypothesis that an endogenous cannabinoid (CB) receptor agonist, such as N-arachidonylethanolamine (anandamide), is the transmitter that mediates perivascular sensory nerve-dependent Ca\(^{2+}\)-induced relaxation. Rat mesenteric branch arteries were studied using wire myography; relaxation was determined after inducing contraction with norepinephrine. Cumulative addition of Ca\(^{2+}\) caused dose-dependent relaxation (ED\(_{50}\) = 2.2 ± 0.09 mM). The relaxation was inhibited by 10 mM TEA and 100 nM iberiotoxin, a blocker of large conductance Ca\(^{2+}\)-activated K\(^+\) channels, but not by 5 \(\mu\)M glibenclamide, 1 mM 4-aminopyridine, or 30 nM apamin. Ca\(^{2+}\)-induced relaxation was also blocked by the selective CB receptor antagonist SR141716A and was enhanced by pretreatment with 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (pefabloc; 30 \(\mu\)M), an inhibitor of anandamide metabolism. Anandamide also caused dose-dependent relaxation (ED\(_{50}\) = .72 ± 0.3 \(\mu\)M). The relaxation was not inhibited by endothelial denudation, 10 \(\mu\)M indomethacin, or 1 \(\mu\)M miconazole, but was blocked by 3 \(\mu\)M SR141716A, 10 mM TEA, precontraction with 100 mM K\(^+\), and 100 nM iberiotoxin, and was enhanced by treatment with 30 \(\mu\)M pefabloc. Mesenteric branch arteries were 200-fold more sensitive to the relaxing action of anandamide than arachidonic acid (ED\(_{50}\) = 160 ± 7 \(\mu\)M). These data show that: 1) Ca\(^{2+}\) and anandamide cause hyperpolarization-mediated relaxation of mesenteric branch arteries, which is dependent on an iberiotoxin-sensitive Ca\(^{2+}\)-activated K\(^+\) channel, 2) relaxation induced by both Ca\(^{2+}\) and anandamide is inhibited by CB receptor blockade, and 3) relaxation induced by anandamide is not dependent on its breakdown to arachidonic acid and subsequent metabolism. These findings support the hypothesis that anandamide, or a similar cannabinoid receptor agonist, mediates nerve-dependent Ca\(^{2+}\)-induced relaxation in the rat.

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ABBREVIATIONS: CaR, receptor for extracellular Ca\(^{2+}\); CB, cannabinoid; 4-AP, 4-aminopyridine; CB\(_1\), type 1 cannabinoid receptor; CB\(_2\), type 2 cannabinoid receptor; CGRP, calcitonin gene-related peptide; K\(_{Ca}\), Ca\(^{2+}\)-activated K\(^+\); NAE, N-arachidonylethanolamine; SNP, sodium nitroprusside.
that was equivalent to an internal diameter of 200 to 225 μm.

Sequential measurement of Ca$^{2+}$-induced relaxation was induced by the addition of 5 μmol/l norepinephrine, and the relaxation effect on subsequently determined responses. The ED50 values were converted to log values for statistical analysis. All data are presented as mean ± S.E.M. and statistical analysis was performed using the SYSTAT software package (SPSS, Inc., Chicago, IL). Comparisons among groups were performed using ANOVA with a repeated measures design when appropriate. A value of p < .05 was taken to indicate a statistically significant difference.

Results

Ca$^{2+}$-Induced Relaxation. We previously demonstrated that nerve dependent, Ca$^{2+}$-induced relaxation can be inhibited by precontraction of arteries with a depolarizing concentration of K+ and by pretreatment with 10 mM TEA. These findings were interpreted to indicate that the relaxation is associated with the release of a hyperpolarizing vasodilator. The present study also assessed the effect of 10 mM TEA and confirmed that it blocks Ca$^{2+}$-induced relaxation (Fig. 1). The effect of four different selective K$^{+}$ channel antagonists was also assessed to pharmacologically characterize the K$^{+}$ channel subtype that is involved in the Ca$^{2+}$-induced relaxation event. Apamin, which is a specific blocker of small conductance Ca$^{2+}$-activated K$^{+}$ (KCa) channels (Murphy and Brayden, 1995), did not affect Ca$^{2+}$-induced relaxation (Fig. 1). Glibenclamide, a selective ATP-sensitive potassium channel antagonist (Meisheri et al., 1993), at a concentration that completely antagonized the relaxation response to pinacidil, also had no effect on Ca$^{2+}$-induced relaxation (Fig. 1). 4-AP, at a dose that has been shown to inhibit voltage-dependent rectifying K$^{+}$ channels (Quayle et al., 1993), was also without effect on Ca$^{2+}$-induced relaxation (Fig. 1). In contrast with these compounds, 100 nM iberiotoxin, which is a concentration that has been shown to block the large-conductance KCa channels (Galvez et al., 1990), completely inhibited relaxation caused by extracellular Ca$^{2+}$ (Fig. 1). These findings indicate that the large-conductance KCa channel is selectively involved in mediating Ca$^{2+}$-induced relaxation of isolated mesenteric resistance arteries.

SR141716A, which is a selective CB receptor antagonist (Rinaldi-Carmona et al., 1994) was used to test the hypothesis that a CB receptor is involved in mediating Ca$^{2+}$-induced relaxation.

Experimental Procedures

Materials. The following compounds were used: norepinephrine, sodium nitroprusside (SNP), arachidonylthanolamide (anandamide), arachidonic acid, pinacidil, TEA, apamin, 4-aminopyridine (4-AP), iberiotoxin, glibenclamide, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (pefabloc), and SR141716A. Pefabloc was purchased from Boehringer Mannheim (Indianapolis, IN) and SR141716A was a generous gift from Dr. Alt-Edmunds (Sanofi Recherche, Montpelier, France). Unless otherwise noted, all other chemicals used in these studies were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals. All procedures involving animals were performed in accordance with approval of the Institutional Animal Care and Use Committee. Male Wistar rats (8–10 weeks of age) were obtained from Harlan Sprague-Dawley and, on arrival in our animal care facility, were maintained in colony rooms with fixed light/dark cycles and constant temperature and humidity and provided with Purina rodent chow and water ad libitum. Mesenteric tissue was isolated while the rats were anesthetized with a mixture of ketamine and xylazine (100:5 mg/kg) and placed in ice-cold physiologic salt solution of the following composition (in mmol/liter): NaCl, 150; KCl, 5.4; MgSO$_4$7H$_2$O, 1.17; NaH$_2$PO$_4$, 1.18; NaHCO$_3$, 6.0; CaCl$_2$, 1.0; HEPES, 20; and glucose, 5.5; pH 7.4.

Biophysical Measurements. Isometric force generation was measured using methods described previously (Bukoski et al., 1997). After isolation, branch II or III mesenteric arteries were cleaned of fat and connective tissue. In some cases, arterial segments were denuded of endothelium by perfusion of the lumen of the vessel with air and subsequent abrasion with a human hair. After cleaning, the vessels were mounted on a wire myograph warmed to 37°C and constant temperature and humidity and provided with Purina rodent chow and water ad libitum. Mesenteric tissue was isolated.

Relaxation to specific compounds was assessed by cumulatively adding the agent to vessels that were precontracted with 5 μmol/l norepinephrine. The magnitude of relaxation was expressed as the difference in isometric force generation caused by extracellular Ca$^{2+}$ (Fig. 1). These findings indicate that the large-conductance KCa channel is selectively involved in mediating Ca$^{2+}$-induced relaxation of isolated mesenteric resistance arteries.

SR141716A, which is a selective CB receptor antagonist (Rinaldi-Carmona et al., 1994) was used to test the hypothesis that a CB receptor is involved in mediating Ca$^{2+}$-induced relaxation.
duced relaxation. SR141716A caused dose-dependent inhibition of Ca^{2+}-induced relaxation with an estimated IC_{50} value of 0.50 μM, and reduced the magnitude of the response to Ca^{2+} by more than 70% (Fig. 2A). At the highest concentration that was used, SR141716A had no effect on pinacidil-induced relaxation, indicating that the compound does not have nonspecific ATP-sensitive potassium channel blocking activity (Fig. 2B). In contrast, pinacidil-induced relaxation was completely antagonized by 5 μM glibenclamide (Fig. 2B). Moreover, treatment of mesenteric arteries with SR141716A from 0.3 to 3 μM was without effect on the magnitude of the force response to 5 μM norepinephrine (control = 1.26 ± 0.2 mN/mm versus 1.04 ± 0.2 mN/mm after 1 μM SR141716A, n = 6–8, p > .05; and control tension = 0.94 ± 0.1 mN/mm versus 0.66 ± 0.1 mN/mm after 3 μM SR141716A, n = 8, p > .05).

Because these data indicate that a CB receptor agonist may be involved in mediating Ca^{2+}-induced relaxation, we assessed the effect of anandamide on precontracted mesenteric branch arteries. Anandamide caused a dose-dependent relaxation of the isolated mesenteric resistance arteries with an ED_{50} value of 0.72 ± 0.3 μM (Fig. 3A). As with the response to Ca^{2+}, the vasodilator effect of anandamide was blocked by pretreatment with 3 μM SR141716A and the magnitude of the blockade was greater at lower concentrations of anandamide; this is consistent with the competitive nature of the antagonist (Fig. 3A). Moreover, when the effect of several different K^{+} channel antagonists was assessed, it was found that anandamide-induced relaxation was blocked by precontraction in depolarizing K^{+}, by pretreatment with 10 mM TEA, and with 100 nM iberiotoxin (Fig. 3B). This pattern of inhibition is identical with that observed for the Ca^{2+}-evoked relaxation event.

The next set of experiments was performed to determine whether, as has been reported for the bovine coronary artery (Pratt et al., 1998), the dilator effect of anandamide might be

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**Fig. 2.** Effect of SR141716A on Ca^{2+}- and pinacidil-induced relaxation. A, Ca^{2+}-induced relaxation was determined under control conditions and after pretreatment with varying concentrations of the CB receptor antagonist SR141716A. Values are mean ± S.E.M.; n = 19 for control, 6 to 8 for experimental. *Indicates a significant inhibitory effect of SR141716A at p < .05; **p < .005. B, relaxation induced by pinacidil in the presence and absence of 0.3 and 3 μM SR141716A or 5 μM glibenclamide. SR141716A was without effect; n = 5 to 6, p = .903 whereas glibenclamide caused significant inhibition at p < .05, n = 4.

**Fig. 3.** A, response of norepinephrine precontracted mesenteric branch arteries to anandamide under control conditions and after pretreatment with 3 μM SR141716A. *Indicates a significant inhibitory effect of SR141716A at p < .05, **p < .005; n = 6. B, response to anandamide after precontraction with 100 mM K^{+}, pretreatment with 10 mM TEA, or 100 nM iberiotoxin. *Indicates a significant difference from control at p < .05, **p < .005; n = 6 to 7.
associated with the breakdown of the amide to arachidonic acid and subsequent metabolism by endothelial cells to another vasodilator compound. Anandamide-induced relaxation was assessed in endothelium-denuded vessel segments to test for a role of the endothelium. Denudation of arteries caused nearly complete ablation of relaxation induced by 1 μM acetylcholine (control relaxation = 75.8 ± 2.8% versus denuded = 2.4 ± 2.0%, p < .001, n = 5–6), but had no effect on anandamide-induced relaxation (Fig. 4). The effect of pretreatment with indomethacin was tested to assess the possible role of cyclooxygenase-generated metabolites of anandamide or arachidonic acid. Indomethacin was also without effect (Fig. 4). The effect of pretreatment with 1 μM miconazole was assessed to determine whether anandamide or a metabolite was converted to a P-450 epoxygenase-generated dilator. Miconazole did not inhibit anandamide-induced relaxation, but caused a slight but significant increase in apparent sensitivity to the compound (Fig. 4).

To provide further insight into the possibility that anandamide might cause relaxation by breakdown to arachidonic acid and subsequent conversion to a vasodilator prostanoid, we assessed the ability of arachidonic acid to relax preconstricted mesenteric branch arteries. Arachidonic acid caused a dose-dependent relaxation, with a maximal relaxation to 31.9 ± 12.6% of initial tension and an ED50 of 0.16 ± 0.07 mM (Fig. 4). This ED50 value for arachidonic acid is nearly 200-fold greater than that for anandamide (0.72 ± 0.3 μM; p < .05, n = 5–6), an observation that makes it highly unlikely that anandamide-induced relaxation is secondary to breakdown to arachidonic acid.

In view of reports that significant breakdown of anandamide can occur in the presence of biological tissue, we assessed the effect of pefabloc, which is an amidase inhibitor comparable to phenylmethylsulfonyl fluoride (Schmid et al., 1985; Pertwee et al., 1995) that significantly attenuates metabolic degradation of anandamide, on Ca2+-induced relaxation. Pefabloc caused a significant increase in the Ca2+-sensitivity of Ca2+-induced relaxation (Fig. 5A; ED50 control = 2.20 ± 0.09 mM versus ED50 pefabloc = 1.75 ± 0.10 mM; n = 6, p < .05). In contrast, the relaxation response to SNP, which should not be altered by an amidase inhibitor, was unaffected by pefabloc (Fig. 5B). When the effect of pefabloc on anandamide-induced relaxation was assessed, the amidase blocker significantly enhanced the relaxation response (Fig. 5C), causing a significant leftward shift in ED50 for anandamide (ED50 control = 0.71 ± 0.2 μM versus ED50 pefabloc = 0.25 ± 0.03 μM; n = 5, p < .05).

**Discussion**

We recently described a perivascular sensory nerve CaR-dependent dilator system that is present in the arterial wall of the rat (Bukoski et al., 1997). In an effort to understand this system more completely, we have now tested the hypothesis that an N-acylphosphatidylethanolamine, such as anandamide, is the hyperpolarizing vasodilator that mediates Ca2+-evoked relaxation. The major new findings of the study are: 1) relaxation induced by both Ca2+ and anandamide are mediated by the opening of the iberiotoxin-sensitive K Ca channel, 2) both Ca2+ - and anandamide-induced relaxation are inhibited by cannabinoid receptor blockade, and 3) anandamide induces relaxation through a direct effect on smooth muscle, and not secondary to breakdown to arachidonic acid and subsequent metabolism to other vasoactive compounds. Collectively, these data support the hypothesis that anandamide, or a related CB receptor agonist, is the hyperpolarizing vasodilator compound that is released by sensory nerves in response to Ca2+ receptor activation.

Anandamide is an endogenous ligand of the CB receptor (Devane et al., 1992; Felder et al., 1993; Fride and Mechoulam, 1993; Crawley et al., 1993; Smith et al., 1994) that has been detected in specific regions of the brain (Di Marzo et al., 1994; Devane and Axelrod, 1994) and in peripheral tissue (Felder et al., 1996; Bisogno et al., 1997; Wagner et al., 1997) and is believed to arise from the phosphodiesterase-mediated release from the precursor molecule N-arachidonoylphosphatidylethanolamine (Di Marzo et al., 1994; Sugiuira et al., 1996). To our knowledge, anandamide has not been chemically isolated from peripheral nerves nor has it previously been proposed to be a transmitter in this system.

Our finding that anandamide causes the relaxation of isolated mesenteric branch arteries supports the early finding of Ellis et al. (1995) who showed, using a cranial window preparation, that anandamide dilates cerebral arteries. Our data

![Fig. 4. Relaxation response of mesenteric branch arteries to anandamide under control conditions, and after removal of endothelium, pretreatment with 10 μM indomethacin or 1 μM miconazole. Shown for comparison is the relaxation response to cumulative addition of arachidonic acid to untreated vessel segments. Values are mean ± S.E.M.; n = 5 for control, 5 to 6 for experimental. *Indicates a difference between relaxation induced by arachidonic acid and the anandamide groups at p < .05; **indicates a significant effect of miconazole at p < .05.](image-url)
also agree with the findings of Randall et al. (1996), who showed that anandamide induces relaxation of the perfused mesenteric bed, and Plane et al. (1997) and White and Hiley (1997), who found that anandamide relaxes the isolated mesenteric branch arteries. Moreover, our finding that anandamide-induced relaxation is \( K_{Ca} \) channel-dependent supports the conclusion of several of these investigators that the dilator acts through a hyperpolarizing mechanism (Randall et al., 1996; Plane et al., 1997, White and Hiley, 1997). In addition to its confirmatory nature, our work provides an important extension of these prior studies by showing that, like \( Ca^{2+} \)-induced relaxation, anandamide-induced relaxation is sensitive to iberiotoxin and thus appears to be mediated by the opening of a large conductance \( KCa \) channel.

Anandamide is believed to exert its biologic actions through the activation of specific CB receptors that are membrane spanning, G protein-coupled proteins. Two distinct CB receptor subtypes have been identified: \( CB_1 \), which is mainly expressed in the brain (Matsuda et al., 1990), and \( CB_2 \), which is located in peripheral tissues, including the lymph system, testis, and kidney (Gerard et al., 1991; Munro et al., 1993; Deutsch et al., 1997). As a test of the hypothesis that a CB receptor/CB ligand system is involved in \( Ca^{2+} \)-induced relaxation, we assessed the effect of SR141716A, which binds to \( CB_1 \) with a \( K_i \) of approximately 10 nM (Felder et al., 1995) and to \( CB_2 \) with a \( K_i \) of approximately 700 nM (Showalter et al., 1996). Our finding that SR141716A blocks \( Ca^{2+} \)-induced relaxation with an estimated \( IC_{50} \) value of 500 nM is consistent with the involvement of a \( CB_2 \)-like receptor. White and Hiley (1997) reached a similar conclusion from their studies, whereas Plane et al. (1997) reported that anandamide-induced relaxation of mesenteric branch arteries was not blocked by SR141716A. The reason for the discrepancy between these studies is unclear.

Of additional importance to the present study was our finding that similar concentrations of SR141716A are required to inhibit both \( Ca^{2+} \) - and anandamide-induced relaxation. This observation supports the idea that anandamide or a similar CB receptor agonist serves as the mediator of \( Ca^{2+} \)-induced relaxation.

Another aspect of this study that warrants discussion is the result of the studies testing the hypothesis that relax-


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