

A Role for *N*-Arachidonylethanolamine (Anandamide) as the Mediator of Sensory Nerve-Dependent Ca^{2+} -Induced Relaxation¹

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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 ($\text{ED}_{50} = 2.2 \pm 0.09$ 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 μM 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 μM), an inhibitor of anandamide metabolism. Anandamide also caused dose-dependent relaxation ($\text{ED}_{50} = .72 \pm 0.3$ μM). The relaxation was not inhibited by

endothelial denudation, 10 μM indomethacin, or 1 μM miconazole, but was blocked by 3 μM SR141716A, 10 mM TEA, precontraction with 100 mM K^+ , and 100 nM iberiotoxin, and was enhanced by treatment with 30 μM pefabloc. Mesenteric branch arteries were 200-fold more sensitive to the relaxing action of anandamide than arachidonic acid ($\text{ED}_{50} = 160 \pm 7$ μM). 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.

We recently demonstrated that perivascular sensory nerve endings express a membrane-spanning receptor for extracellular Ca^{2+} (CaR; Bukoski et al., 1997; Mupanomunda et al., 1998) that is homologous to the CaR expressed in the parathyroid (Brown et al., 1993), kidney (Riccardi et al., 1995), and brain (Ruat et al., 1995). We also showed that stimulation of isolated arteries with physiologic concentrations of extracellular Ca^{2+} causes vasodilation that is unaffected by endothelial denudation, but is sensory nerve-dependent and associated with the release of a vasodilator from the periaxonal surface of the artery (Bukoski et al., 1997; Mupanomunda et al., 1998). The relaxation is completely blocked by precontraction of the arterial segments with depolarizing concentrations of K^+ , and during nonselective blockade of K^+ channels with TEA (Bian et al., 1995; Bukoski et al., 1997). The relaxation is not, however, affected by pharmacologic

blockade of receptors for known sensory peptide neurotransmitters, i.e., calcitonin gene-related peptide, Substance P, and neurokinin A. These observations, and more recent data obtained using in situ microdialysis-based measurements of interstitial Ca^{2+} in the duodenal submucosa (Mupanomunda and Bukoski, 1998), have led us to propose that the periaxonal nerve CaR is activated by dynamic changes in interstitial Ca^{2+} that occur during transcellular Ca^{2+} movement, and that activation of the receptor is coupled to the release of a nonpeptide-hyperpolarizing dilator (Bukoski et al., 1997).

One group of hyperpolarizing vasodilator compounds that have been isolated from neural tissue are the *N*-acylethanolamines (NAEs), which are endogenous cannabinoid receptor agonists. These compounds, which were originally isolated from brain, mimic many of the behavioral responses of Δ^9 -tetrahydrocannabinol, the main psychotropic component of the medicinal herb *Cannabis sativa*. The responses include analgesia, catalepsy, and hypothermia (Felder et al., 1993;

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ABBREVIATIONS: CaR, receptor for extracellular Ca^{2+} ; CB, cannabinoid; 4-AP, 4-aminopyridine; CB₁, type 1 cannabinoid receptor; CB₂, type 2 cannabinoid receptor; CGRP, calcitonin gene-related peptide; K_{Ca} , Ca^{2+} -activated K^+ ; NAE, *N*-acylethanolamine; SNP, sodium nitroprusside.

Fride and Mechoulam, 1993; Crawley et al., 1993; Smith et al., 1994). The best studied NAE to date, *N*-arachidonyl ethanolamine (or anandamide), has also been shown to lower blood pressure in the conscious rat (Varga et al., 1996; Lake et al., 1997) and to cause relaxation of isolated preparations of vascular smooth muscle (Randall et al., 1996; Plane et al., 1997; White and Hiley, 1997). In view of the neuronal origin of the NAEs, and reports that these compounds are hyperpolarizing vasodilators, we performed experiments that tested the hypothesis that an *N*-acyl-ethanolamine, such as anandamide, serves as a transmitter in the perivascular sensory nerve-mediated, extracellular Ca^{2+} -activated dilator system.

Experimental Procedures

Materials. The following compounds were used: norepinephrine, sodium nitroprusside (SNP), arachidonyl ethanolamide (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, Montpellier, 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; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.17; NaH_2PO_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 gassed with 95% air/5% CO_2 , stretched to a predetermined length that was equivalent to an internal diameter of 200 to 225 μm , and allowed to equilibrate for 30 min. After the equilibration period, the vessels were induced to contract with 5 μM norepinephrine until reproducible contractile responses were obtained (three-four times).

Relaxation to specific compounds was assessed by cumulatively adding the agent to vessels that were precontracted with 5 μM norepinephrine. The magnitude of relaxation was expressed as the percentage of the initial tension. When phasic activity was present, measurements were taken from the precontraction baseline to the trough of the phasic transition. When the effect of a specific antagonist on Ca^{2+} -induced relaxation was assessed, the vessel was pretreated with the compound for 15 min, after which the contraction was induced by the addition of 5 $\mu\text{mol/l}$ norepinephrine, and the response to the dilator assessed. In some experiments the effect of a given antagonist on Ca^{2+} -induced relaxation was assessed after determining the control response to Ca^{2+} . Control experiments showed that sequential measurement of Ca^{2+} -induced relaxation had no effect on subsequently determined responses.

Statistical Analysis. The ED_{50} was determined by linear regression analysis by use of the data points immediately above and below the half-maximal response. The ED_{50} values were converted to log values for statistical analysis. All data are presented as mean $\pm$

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^+ (K_{Ca}) 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 K_{Ca} channels (Galvez et al., 1990), completely inhibited relaxation caused by extracellular Ca^{2+} (Fig. 1). These findings indicate that the large-conductance K_{Ca} 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+} -in-

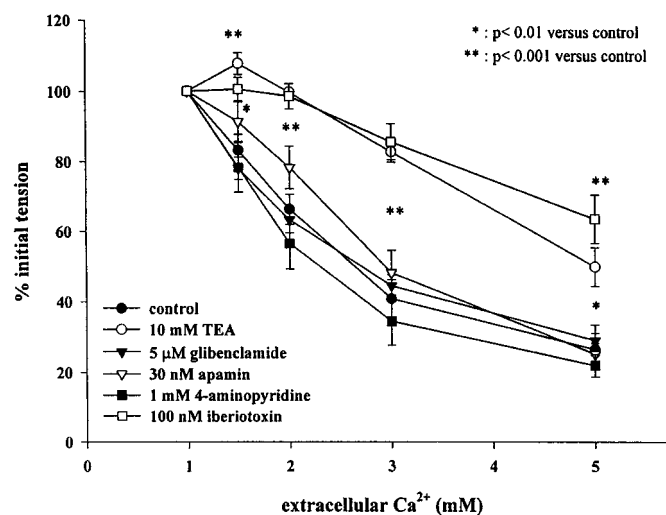


Fig. 1. Effect of K^+ channel inhibition on Ca^{2+} -induced relaxation. Ca^{2+} -induced relaxation was determined in mesenteric branch arteries precontracted with 5 μM norepinephrine after pretreatment with 10 mM TEA, 5 μM glibenclamide, 1 mM 4-AP, 30 nM apamin, and 100 nM iberiotoxin. Values are mean $\pm$ S.E.M.; $n = 7$ to 9. *Indicates a significant inhibitory effect of the blocker at $p < .01$; at $**p < .001$.

duced relaxation. SR141716A caused dose-dependent inhibition of Ca^{2+} -induced relaxation with an estimated IC_{50} value of $0.50 \mu\text{M}$, 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 $5 \mu\text{M}$ glibenclamide (Fig. 2B). Moreover, treatment of mesenteric arteries with SR141716A from 0.3 to $3 \mu\text{M}$ was without effect on the magnitude of the force response to $5 \mu\text{M}$ norepinephrine (control = 1.26 ± 0.2 mN/mm versus 1.04 ± 0.2 mN/mm after $1 \mu\text{M}$ SR141716A, $n = 6-8$, $p > .05$; and control tension = 0.94 ± 0.1 mN/mm versus 0.66 ± 0.1 mN/mm after $3 \mu\text{M}$ 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 $0.72 \pm 0.3 \mu\text{M}$ (Fig. 3A). As with the response to Ca^{2+} , the vasodilator effect of anandamide was blocked by pretreatment with $3 \mu\text{M}$ 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

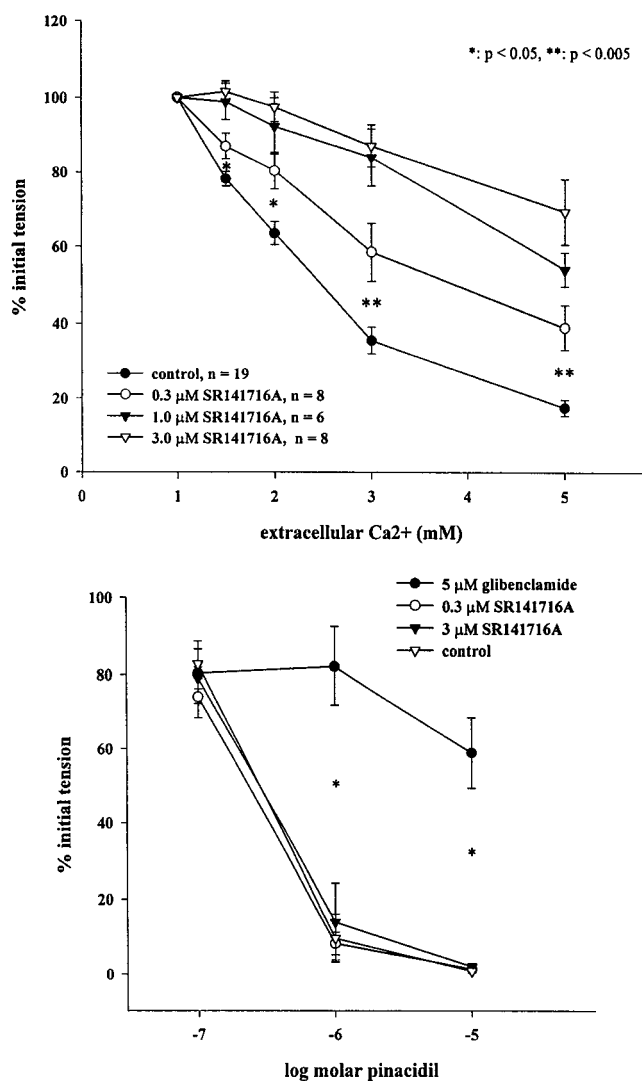


Fig. 2. Effect of SR141716A on Ca^{2+} - and pinacidil-induced relaxation. A, Ca^{2+} -induced relaxation was determined under control conditions and after pretreatment with varying concentrations of the CB₁ antagonist SR141716A. Values are mean $\pm$ S.E.M.; $n = 19$ for control, 6 to 8 for experimental. *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 $3 \mu\text{M}$ SR141716A or $5 \mu\text{M}$ glibenclamide. SR141716A was without effect; $n = 5$ to 6 , $p = .903$ whereas glibenclamide caused significant inhibition at $p < .05$, $n = 4$.

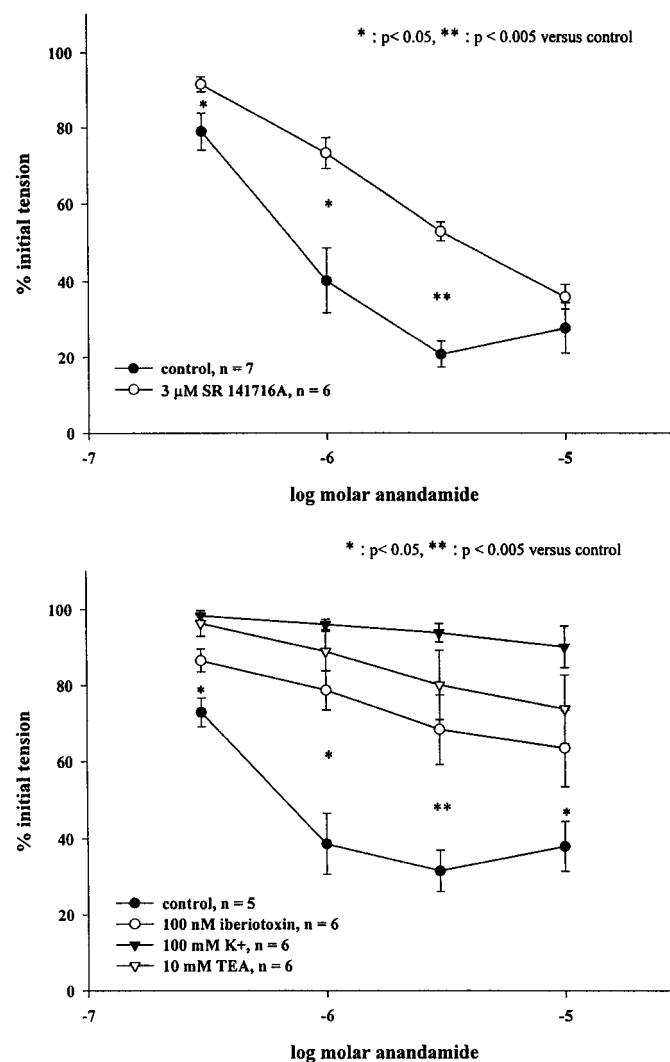


Fig. 3. A, response of norepinephrine precontracted mesenteric branch arteries to anandamide under control conditions and after pretreatment with $3 \mu\text{M}$ SR141716A. *Indicates a significant inhibitory effect of SR141716A at $p < .05$, ** $p < .005$; $n = 6$. B, response to anandamide after precontraction 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 μ M acetylcholine (control relaxation = $75.8 \pm 2.8\%$ versus denuded = $2.4 \pm 2.0\%$, $p < .001$, $n = 5-6$), 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 μ M miconazole was assessed to determine whether anandamide or a metabolite was converted to a P-450 epoxigenase-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 precontracted mesenteric branch arteries. Arachidonic acid caused a dose-dependent relaxation, with a maximal relaxation to $31.9 \pm 12.6\%$ of initial tension and an ED_{50} of 0.16 ± 0.07 mM (Fig. 4). This ED_{50} value for arachidonic acid is nearly 200-fold greater than that for anandamide (0.72 ± 0.3 μ M; $p < .05$, $n = 5-6$), 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 Ca^{2+} -induced relaxation. Pefabloc caused a significant increase in the Ca^{2+} -sensitivity of Ca^{2+} -induced relaxation (Fig. 5A; ED_{50} control = 2.20 ± 0.09 mM versus ED_{50} 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 ED_{50} for anandamide (ED_{50} control = 0.71 ± 0.2 μ M versus ED_{50} pefabloc = 0.25 ± 0.03 μ M; $n = 5$, $p < .05$).

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*-acylethanolamine, such as anandamide, is the hyperpolarizing vasodilator that mediates Ca^{2+} -evoked relaxation. The major new findings of the study are: 1) relaxation induced by both Ca^{2+} and anandamide are mediated by the opening of the iberiotoxin-sensitive K_{Ca} channel, 2) both Ca^{2+} - 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 Ca^{2+} receptor activation.

Anandamide is an endogenous ligand of the CB receptor (Devane et al., 1992; Felder et al., 1993; Frider 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*-arachidonylethanolamine (Di Marzo et al., 1994; Sugiura 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

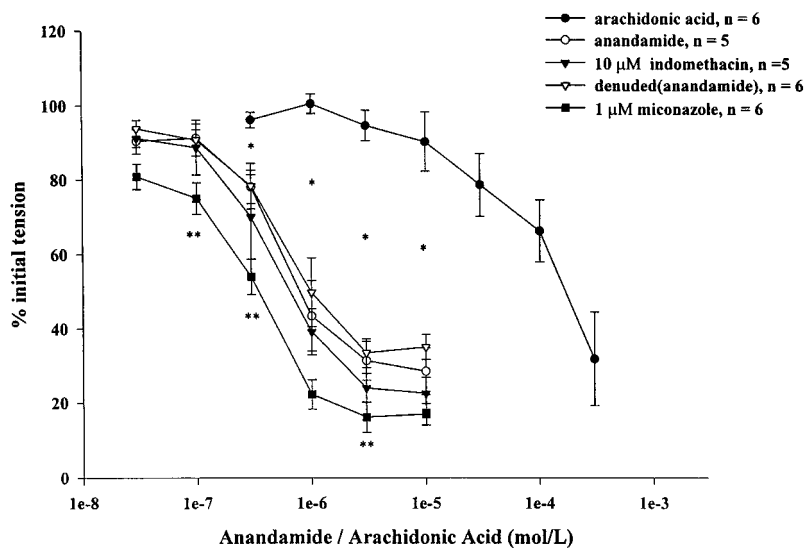


Fig. 4. Relaxation response of mesenteric branch arteries to anandamide under control conditions, and after removal of endothelium, pretreatment with 10 μ M indomethacin or 1 μ M miconazole. Shown for comparison is the relaxation response to cumulative addition of arachidonic acid to untreated vessel segments. Values are mean $\pm$ 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 $p < .05$.

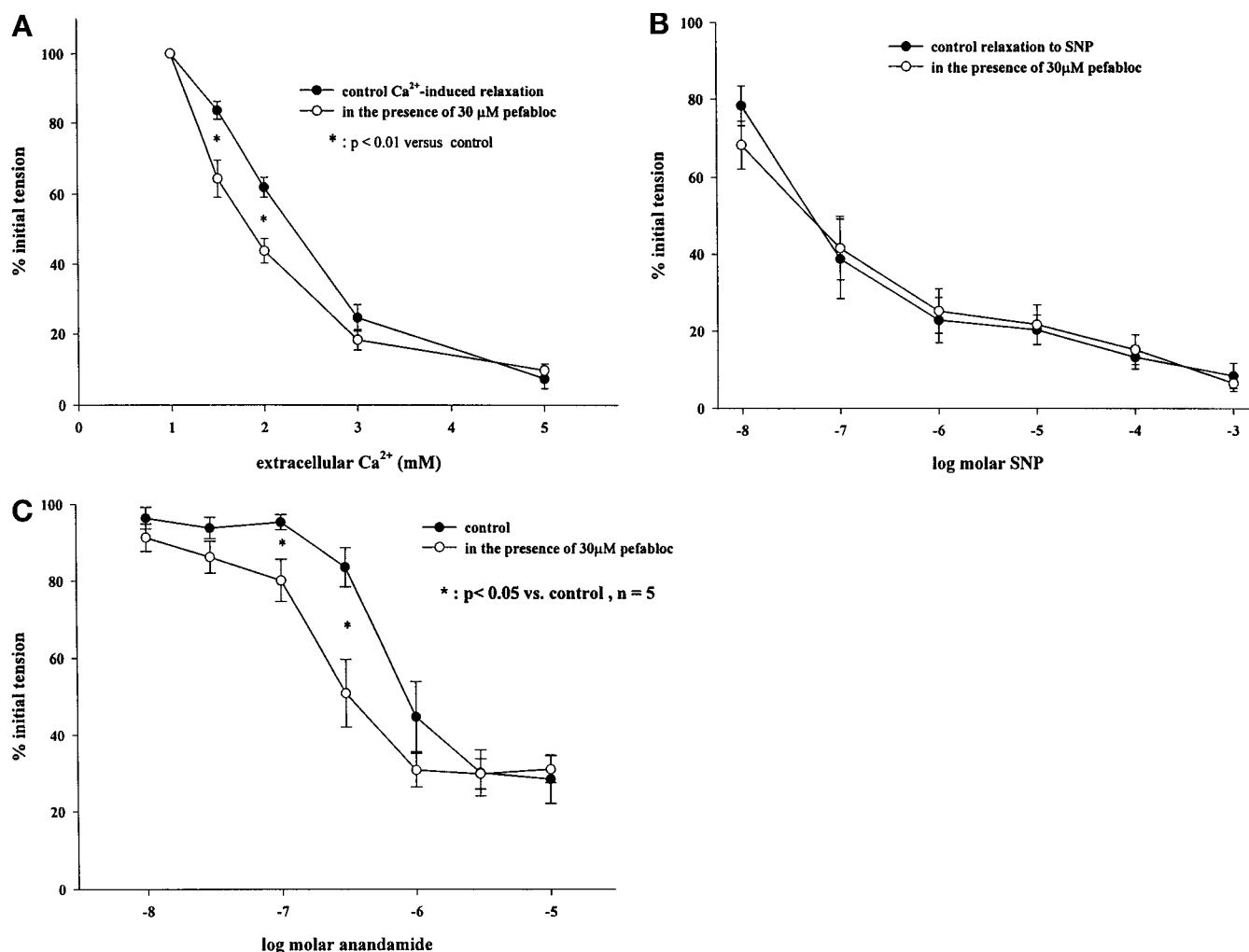


Fig. 5. Effect of amidase inhibition on Ca^{2+} , SNP, and anandamide-induced relaxation. **A**, Ca^{2+} -induced relaxation was determined before and after 10 min incubation with 30 μM pefabloc. *Indicates pefabloc caused a significant leftward shift in the concentration response to Ca^{2+} (control ED_{50} for Ca^{2+} = 2.20 ± 0.09 mM versus 1.75 ± 0.10 mM during exposure to pefabloc, $n = 5$ to 6 , $p < .05$). **B**, SNP-induced relaxation was determined under control conditions and after pretreatment with 30 μM pefabloc. Pefabloc had no effect on SNP-induced relaxation ($n = 5$ to 6 , $p = .965$). **C**, anandamide-induced relaxation before and after treatment with 30 μM pefabloc. *Pefabloc significantly decreased the relaxation response and caused a leftward shift in sensitivity to anandamide (ED_{50} control = 0.71 ± 0.2 μM versus ED_{50} pefabloc = 0.25 ± 0.03 μM ; $n = 5$, $p < .05$).

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^+ 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 ibuprofen and thus appears to be mediated by the opening of a large conductance K_{Ca} 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-

ation induced by anandamide is not elicited by anandamide, but by a metabolite of this compound. These experiments were prompted by two different observations. One is the fact that anandamide can be degraded to arachidonic acid and ethanolamine by endogenous amidase activity (Bisogno et al., 1997). The second is the report of Pratt et al. (1998), which showed using the bovine coronary artery that anandamide-induced relaxation is not linked with activation of the CB receptor, but is dependent on its breakdown to arachidonic acid and subsequent metabolism by cyclooxygenase and P-450 epoxigenase enzymes to other dilator products. Three lines of evidence indicate that this series of events does not occur in the rat mesenteric branch artery: 1) the finding that anandamide-induced relaxation is antagonized by CB receptor blockade, 2) the observation that the sensitivity of mesenteric branch arteries to arachidonic acid is 200-fold less than to anandamide, and 3) the finding that anandamide-induced relaxation is unaffected by endothelial denudation, or by blockade of the cyclooxygenase or cytochrome P-450 epoxigenase pathways. In view of these findings, we conclude that, unlike the cow arteries, anandamide causes relaxation of the isolated rat mesenteric branch artery through a direct action of the native compound. The difference in the two studies could reflect variations associated with either the anatomical source of the arteries or with species differences.

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