CB₁ Receptor Antagonist SR141716A Inhibits Ca²⁺-Induced Relaxation in CB₁ Receptor–Deficient Mice

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Abstract—Mesenteric branch arteries isolated from cannabinoid type 1 receptor knockout (CB₁⁻/⁻) mice, their wild-type littermates (CB₁⁺/⁺ mice), and C57BL/6J wild-type mice were studied to test the hypothesis that murine arteries undergo high sensitivity Ca²⁺-induced relaxation that is CB₁ receptor dependent. Confocal microscope analysis of mesenteric branch arteries from wild-type mice showed the presence of Ca²⁺ receptor–positive periadventitial nerves. Arterial segments of C57 control mice mounted on wire myographs contracted in response to 5 µmol/L norepinephrine and responded to the cumulative addition of extracellular Ca²⁺ with a concentration-dependent relaxation that reached a maximum of 72.0±6.3% of the prerelaxation tone and had an EC₅₀ for Ca²⁺ of 2.90±0.54 mmol/L. The relaxation was antagonized by precontraction in buffer containing 100 mmol/L K⁺ and by pretreatment with 10 mmol/L tetraethylammonium. Arteries from CB₁⁻/⁻ and CB₁⁺/⁺ mice also relaxed in response to extracellular Ca²⁺ with no differences being detected between the knockout and their littermate controls. SR141716A, a selective CB₁ antagonist, caused concentration-dependent inhibition of Ca²⁺-induced relaxation in both the knockout and wild-type strains (60% inhibition at 1 µmol/L). O-1918, a cannabidiol analog, had a similar blocking effect in arteries of both wild-type and CB₁⁻/⁻ mice at 10 µmol/L. In contrast, 1 µmol/L SR144538, a cannabinoid type 2 receptor antagonist, or 50 µmol/L 18α-glycyrrhetinic acid, a gap junction blocker, were without effect. SR141716A (1 to 30 µmol/L) was also assessed for nonspecific actions on whole-cell K⁺ currents in isolated vascular smooth muscle cells. SR141716A inhibited macroscopic K⁺ currents at concentrations higher than those required to inhibit Ca²⁺-induced relaxation, and appeared to have little effect on currents through large conductance Ca²⁺-activated K⁺ channels. These data indicate that arteries of the mouse relax in response to cumulative addition of extracellular Ca²⁺ in a hyperpolarization-dependent manner and rule out a role for CB₁ or CB₂ receptors in this effect. The possible role of a nonclassical cannabinoid receptor is discussed. (Hypertension. 2002;39:251-257.)

Key Words: mesenteric arteries ■ calcium ■ relaxation ■ potassium channels

Over the past several years, a novel, extracellular Ca²⁺-activated, vasodilator pathway has been described in isolated rat arteries. The current model describing this system holds that elevation of extracellular Ca²⁺, as occurs in the interstitial compartments of some tissues, activates a Ca²⁺-sensing receptor localized on perivascular sensory nerves. Activation of this receptor causes the release of a transmitter that diffuses to underlying vascular smooth muscle and induces hyperpolarization-mediated relaxation through activation of a vascular smooth muscle cannabinoid type 1 (CB₁) receptor.

Among the findings that support this pathway are the demonstration that dorsal root ganglia and perivascular sensory nerves express a protein that is immunoreactive with antibodies raised against the parathyroid Ca²⁺ receptor and that the magnitude of Ca²⁺-induced relaxation positively correlates with Ca²⁺ receptor–positive nerve density. The relaxation is inhibited by nonselective antagonism of potassium (K⁺) channel activity with precontraction in depolarizing concentrations of KCl and pretreatment with tetraethylammonium and by selective blockade of large conductance Kₐ channels with iberiotoxin and charybotoxin. Additional work has shown that the relaxation is blocked by the selective CB₁ receptor antagonist SR141716A and is mimicked by anandamide, an endocannabinoid, which has been shown to be formed in neuronal tissue.

Over the past 2 years, several reports have appeared that indicate that in addition to its high potency as a CB₁ receptor antagonist, at concentrations of ≥10 µmol/L, SR141716A also has activity as an antagonist of type 2 cannabinoid (CB₂) receptors, Ca²⁺ entry channels, Kₐ channels, and gap junctions. Although we have found that lower concentrations of SR141716A inhibit the mesenteric vasodilator re-

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response to anandamide, we believed it was important to obtain additional information regarding whether Ca\(^{2+}\)-induced relaxation is mediated by the CB\(_1\) receptor. The CB\(_1\) mouse offers a direct means of testing the hypothesis that Ca\(^{2+}\)-induced relaxation is mediated by a transmitter with activity at the CB\(_1\) receptor. We have therefore performed experiments to (1) determine whether arteries isolated from mice undergo extracellular Ca\(^{2+}\)-induced relaxation and whether the relaxation shares properties with the relaxation observed in rats and (2) to test the hypothesis that Ca\(^{2+}\)-induced relaxation is mediated by the CB\(_1\) receptor.

**Methods**

**Animals**

All experiments using animals were approved by the appropriate institutional animal care and use committees. CB\(_1\) receptor knockout (CB\(_1\)^\text{−−}^\text{−}^\text{−}\) and homozygous littermate controls (CB\(_1\)^\text{++}^\text{++}\)) were bred from founders that were generously provided by Dr Andreas Zimmer (Rheinische Friedrich-Wilhelms-Universitat Klinik und Poliklinik fur Psychiatrie und Psychotherapie Abteilung Molekulare Neurobiologie, Bonn, Germany) and that have been described in detail. Some control experiments were performed using tissue obtained from nonlittermate inbred C57BL/6 mice (Harlan Sprague Dawley, Indianapolis, Ind). Male and female animals were studied, and mesenteric tissue was isolated after the animals were killed by asphyxiation followed by cervical dislocation. Male golden Syrian hamsters (9 to 12 weeks of age) were obtained from Charles Rivers Laboratories (Wilmington, Delaware) and on the day of the experiment were killed by CO\(_2\) cervical dislocation. Male and female animals were studied, obtained from nonlittermate inbred C57BL/6 mice (Harlan Sprague Dawley, Indianapolis, Ind). Male and female animals were studied, and mesenteric tissue was isolated after the animals were killed by cervical dislocation. Male golden Syrian hamsters (9 to 12 weeks of age) were obtained from Charles Rivers Laboratories (Wilmington, Delaware) and on the day of the experiment were killed by CO\(_2\) asphyxiation followed by cervical dislocation. Cremaster muscles were removed, and second- and third-order arterioles were isolated by hand dissection as described previously.

**Immunocytochemistry**

Mesenteric branch arteries were microdissected from isolated mesenteries and prepared for immunostaining as described previously. Fixed and blocked segments were incubated overnight with a polyclonal anti-Ca\(^{2+}\) receptor antibody (1:50, Affinity Bioreagents) in the presence or absence of excess antigen. Anti-rabbit IgG conjugated with Texas Red (1:300, Molecular Probes) was used as the secondary antibody. Vessel segments were viewed using a Zeiss LSM 510 confocal microscope and 63\(\times\) water immersion objective.

**Biophysical Measurements**

Isometric force generation was measured using previously described methods. Relaxation to Ca\(^{2+}\) or acetylcholine was assessed by cumulatively adding the agent to vessels that had been precontracted to a steady-state tension using 5 \(\mu\)mol/L norepinephrine (NE). The percentage of relaxation was calculated taking the magnitude of the prerelaxation tone as 100% of the amount that the vessel could relax.

When the effect of an antagonist, ie, SR141716A, was assessed on the relaxation response, the vessel was pretreated with the compound for 10 to 15 minutes. Afterward, the contraction was induced by the addition of NE, and the response to the dilator assessed. An expanded Biophysical Measurements section can be found in an online data supplement available at http://www.hypertensionaha.org.

**Electrophysiology**

Vascular smooth muscle cells were enzymatically isolated from hamster cremasteric arterioles using a papain digestion medium as described previously. Macroscopic K\(^{+}\) currents were measured using the conventional whole cell recording method. Currents were measured, and membrane potential was clamped with a Warner PC-505A patch-clamp amplifier (Warner Instrument Corp), filtered at 1 kHz, and sampled at 5 kHz. As reported previously, all currents were normalized to cell capacitance to account for any differences in cell size. Cells were held at −60 mV and then stepped, for 400 ms, to test potentials from −90 to 60 mV in 10-mV increments. The average current during the last 200 ms of the test pulse was then measured at each potential and used to construct current/voltage relationships.

An expanded Electrophysiology section can be found in an online data supplement available at http://www.hypertensionaha.org.

**Materials**

SR141716A and SR144538 were obtained from Sanofi Recherche and dissolved in absolute ethanol; O-1918 was synthesized by Dr Raj Razdan (Organix Inc, Woburn, Mass) and dissolved in ethanol. All other chemicals were of reagent grade or better and obtained from Sigma Chemical or other commercial suppliers, as dictated by the purchasing regulations of the University of North Carolina System.

**Statistical Analysis**

The agonist concentration eliciting 50% of the maximal relaxation (EC\(_{50}\)) was determined from plots of the percentage of initial tension versus the concentration of agonist. All data are presented as mean±SEM, and statistical analysis was performed using the SYSTAT software package. Comparisons among groups were performed using ANOVA with a repeated measures design when appropriate. A value of \(P<0.05\) was taken to indicate a statistically significant difference.

**Results**

Whole-mount sections of mouse mesenteric branch arteries were stained with anti-Ca\(^{2+}\) receptor antibody and viewed using a laser confocal microscope (Figure 1). Analysis of sections from at least 6 mice showed that nerve fibers located in the adventitia stained positively for Ca\(^{2+}\) receptor protein, and Ca\(^{2+}\) receptor–positive staining was not present elsewhere in the vessel wall (Figure 1A and 1B). Moreover, preincubation of the primary antibody with blocking peptide
or staining with secondary antibody alone eliminated nerve staining (Figure 1C).

Mesenteric branch arteries isolated from C57 control mice and precontracted with 5 μmol/L NE relaxed in response to cumulative addition of extracellular Ca^{2+}, with a maximal relaxation response of 72.0±6.3% of the prerelaxation tone (n=6) and an EC_{50} for Ca^{2+} of 2.9±0.54 mmol/L (Figure 2A).

To learn whether Ca^{2+}-induced relaxation of mouse arteries is dependent on functional K^{+} channels, the relaxation response was also determined in vessels precontracted with a depolarizing concentration of K^{+} and pretreated with 10 mmol/L TEA or a mixture of 10 mmol/L TEA and 0.3 mmol/L NG-nitro-L-arginine methyl ester (L-NAME). Values are mean±SEM, *P<0.05 vs NE control, n=3 to 5 per group.

Branch arteries isolated from CB_{1}^{−/−} animals and CB_{1}^{+/−} littermates were also studied. Ca^{2+} caused a concentration-dependent relaxation of CB_{1}^{−/−} arteries that was not different from that observed in vessels isolated from CB_{1}^{+/−} control
animals (maximal relaxation response, 75.5±6.9%,  
P=0.954; EC_{50}, 3.2±0.28 mmol/L, n=6,  
P=0.714) (Figure 3A). The response of CB_{1}^{+/+} and CB_{1}^{−/−} arteries to the  
cumulative addition of acetylcholine was also assessed. Acetylcholine induced concentration-dependent relaxation of  
arteries isolated from both the wild-type and knockout strains (data not shown). No differences in either the maximal  
response or the sensitivity of the arteries to acetylcholine were detected (maximal CB_{1}^{+/+} response versus CB_{1}^{−/−},  
76.9±7.5% versus 78.3±10.7%, n=3 and n=4, respectively,  
P=0.998; EC_{50} CB_{1}^{+/+} versus CB_{1}^{−/−}, 0.71±0.42 versus  
0.21±0.15 μmol/L,  
P=0.888).

Preincubation of vessels with increasing concentrations of the CB_{1} antagonist SR141716A dose-dependently inhibited  
Ca^{2+}-induced relaxation of NE-precontracted arteries isolated from both the wild-type and knockout strains (data not shown). No differences in either the maximal response or the sensitivity of the arteries to acetylcholine were detected (maximal CB_{1}^{+/+} response versus CB_{1}^{−/−}, 76.9±7.5% versus 78.3±10.7%, n=3 and n=4, respectively,  
P=0.998; EC_{50} CB_{1}^{+/+} versus CB_{1}^{−/−}, 0.71±0.42 versus 0.21±0.15 μmol/L,  
P=0.888).

To learn whether the blockade by SR141716A might be mediated by antagonist activity at the CB_{2} receptor, we assessed the effect of pretreatment with the selective CB_{2} antagonist SR144538. Pretreatment with 1 μmol/L SR144538 had no effect on relaxation induced by Ca^{2+} in arteries isolated from either the knockout or wild-type animals (Figure 4A). To assess the possible role of gap junctional activity in the relaxation response, the effect of preincubation with 18α-glycyrhetinic acid (50 μmol/L), a gap junction blocker was assessed. 18α-glycyrhetinic acid had no effect on the relaxation response determined in vessels from 4 animals (Figure 4B). Our previous data indicate that Ca^{2+}-induced relaxation is inhibited by iberiotoxin, a selective inhibitor of large conduct K_{Ca} channels. To learn whether a possible nonselective effect of SR141716A at K^{+} channels plays a role in the inhibition of Ca^{2+}-induced relaxation, we assessed the effect of a range of concentrations of SR141716A on whole-cell K^{+} channel currents in isolated vascular smooth muscle cells. SR141716A inhibited macroscopic K^{+} currents in a concentration-dependent fashion, (Figure 5A), with maximal effects observed at 10 μmol/L (Figure 5B). To learn whether SR141716A blocks K_{Ca} channel activity, we tested whether the inhibitory action of the
antagonist was additive with respect to TEA. Initial experiments showed that TEA (1 mmol/L) and iberiotoxin (100 nmol/L) inhibit whole-cell K⁺ channel currents to a similar degree and in a nonadditive fashion (Figure 6A). In contrast, the K⁺-blocking effect of SR141716A was clearly additive to the inhibition produced by 1 mmol/L TEA (Figure 6B and 6C) or 100 nmol/L iberiotoxin (n = 3, data not shown). These observations suggest that SR141716A has little effect on currents through large conductance KCa channels.

A previous report from one of our laboratories (G.K.) showed that abnormal-cannabidiol induces relaxation of the isolated perfused mesenteric artery bed of the rat and mouse and that the relaxation is blocked by cannabidiol and by SR141716A. We therefore assessed the effect of a novel cannabidiol analog, O-1918, that has been found to block abnormal cannabidiol-induced relaxation in isolated rat arteries (S. Batkai and G. Kunos, 2001, unpublished observations). Pretreatment of arteries isolated from C57BL/6 wild-type mice and CB₁/⁻/⁻ mice with 10 μmol/L O-1918 for 15 minutes caused significant inhibition of Ca²⁺-induced relaxation (Figure 7A and 7B), whereas it was without effect on relaxation induced by acetylcholine (Figure 7C).

### Discussion

The present studies were performed to determine whether arteries isolated from mice undergo extracellular Ca²⁺-induced relaxation, to learn whether the relaxation shares properties with Ca²⁺-induced relaxation observed in rats, and to test the hypothesis that Ca²⁺-induced relaxation is mediated by the CB₁ receptor. The major new findings are that (1) murine mesenteric branch arteries are supplied with Ca²⁺-receptor–positive periadventitial nerves and relax in response to the cumulative addition of extracellular Ca²⁺; (2) the relaxation is blocked by conditions that inhibit K⁺ channel–dependent relaxation; (3) the relaxation occurs in the absence of CB₁ receptors; and (4) the relaxation is antagonized by SR141716A in arteries of the CB₁ receptor knockout mouse.

Earlier studies, performed using arteries isolated from the rat, showed that when care is taken to preserve the integrity of the vascular adventitia, cumulative addition of extracellular Ca²⁺ causes concentration-dependent relaxation. In recent studies of arterial segments mounted on 14-μm gold wires, we have found EC₅₀ values for Ca²⁺ of 1.45 ± 0.02 mmol/L (R.D. Bukoski, 2001, unpublished observations), which is well within the range of the concentrations that have been shown to occur in the interstitial fluid compartment of the small bowel and renal cortex. The relaxation is attenuated by sensory denervation and correlates with the presence of Ca²⁺ receptor–positive nerve fibers. Ca²⁺-induced relaxation of rat arteries has also been shown to be inhibited by blockers of the large conductance KCa channel and by SR141716A, a compound with CB₁ antagonist activity. Moreover, the relaxation is mimicked by anandamide, an endocannabinoid CB₁ receptor agonist with hyperpolarizing vasodilator activity. Together, these observations have led to the hypothesis that changes in the concentration of Ca²⁺ in the interstitial fluid compartment are sensed by a sensory nerve Ca²⁺ receptor, and activation of this receptor is coupled with
Dorsal root ganglia of the rat express cDNA encoding a protein. These findings extend our prior demonstration that diol analog O-1918 on Ca\(^{2+}\) mouse mesenteric branch arteries express Ca\(^{2+}\) dilator transmitter with activity at the CB<sub>1</sub> receptor.\(^1\) The release of an endocannabinoid-like hyperpolarizing vasoconstrictor transmitter with activity at the CB<sub>1</sub> receptor is the first demonstration of Ca\(^{2+}\) dependent mechanism of relaxation. To our knowledge, this is the first demonstration of Ca\(^{2+}\)-induced relaxation of a species other than the rat.

The second new finding of the present study is the observation that Ca\(^{2+}\)-induced relaxation occurs in mesenteric branch artery segments isolated from CB<sub>1</sub> receptor knockout mice. These experiments were designed to follow up on the prior observation that Ca\(^{2+}\)-induced relaxation in the rat is blocked by SR141716A and to directly test the hypothesis that Ca\(^{2+}\)-induced relaxation requires an active CB<sub>1</sub> receptor. The results clearly indicate that the CB<sub>1</sub> receptor is not involved in the Ca\(^{2+}\)-induced relaxation event in the mouse.

A third finding that merits discussion is our observation that Ca\(^{2+}\)-induced relaxation of mouse artery is inhibited by SR141716A. This supports our earlier finding obtained using the rat.\(^4\) In addition, the observation that the sensitivity of Ca\(^{2+}\)-induced relaxation to inhibition by SR141716A is similar in arteries isolated from the CB<sub>1</sub>-/- knockout and wild-type control argues against the possibility that SR141716A inhibits Ca\(^{2+}\)-induced relaxation via CB<sub>1</sub> receptors in wild-type mice, but suggests that a different site is involved in the inhibition observed in CB<sub>1</sub> receptor knockout mice.

As noted in the Introduction, one possible explanation for our finding that Ca\(^{2+}\)-induced relaxation is intact in the CB<sub>1</sub> knockout mouse, yet is blocked by SR141716A, is that the blockade by SR141716A is mediated by nonspecific actions of the compound. As noted above, it has been reported that at high micromolar concentrations, SR141716A blocks CB<sub>2</sub> receptors,\(^8\) gap junctions,\(^10\) Ca\(^{2+}\) entry channels,\(^10\) and K<sub>Ca</sub> channels.\(^10\) Our finding that SR144538, a selective CB<sub>2</sub> antagonist, had no effect on Ca\(^{2+}\)-induced relaxation in the CB<sub>1</sub> knockout animal indicates that SR141716A is not blocking the relaxation through a CB<sub>1</sub> receptor effect. Moreover, our finding that 18α-glycyrrhetinic acid, a compound with gap junction–blocking activity,\(^10\) has no effect on Ca\(^{2+}\) relaxation in the rat makes it unlikely that SR141716A is attenuating Ca\(^{2+}\)-induced relaxation through a gap junction–blocking effect. It also seems unlikely that SR141716A is blocking Ca\(^{2+}\)-induced relaxation by inhibiting L-type Ca\(^{2+}\) channels because L-type Ca\(^{2+}\) channel inhibition is generally associated with a decrease in agonist-induced contraction and not blockade of a relaxation event.

In view of the fact that Ca\(^{2+}\)-induced relaxation is antagonized by the selective large conductance K<sub>Ca</sub> channel antagonist iberiotoxin,\(^4\) we were particularly concerned about the contribution of possible K<sup></sup> channel–blocking activity of SR1416171A, as has been reported by White and Hiley.\(^9\) To
directly address this issue, we determined the effect of a range of concentrations of SR141716A on whole-cell K⁺ channel currents in isolated vascular smooth muscle cells. The results showed clearly that SR141716A causes concentration-dependent inhibition of these currents. Two findings are of particular interest for the present study. One is that 1 μmol/L SR141716A caused <8% inhibition of K⁺ channel current, whereas the same concentration inhibited Ca²⁺-induced relaxation by >60%. A second finding is that the effect of SR141716A is additive to the effect of TEA and iberiotoxin. This suggests that SR141716A inhibits K⁺ channels other than Kᵥ channels, presumably voltage-dependent K⁺ channels, which are less sensitive to SR141716A than the site at which SR141716A inhibits Ca²⁺-induced relaxation. In view of these observations, it seems unlikely that the inhibitory effect of SR141716A on Ca²⁺-induced relaxation is mediated by an inhibition of either Kᵥ or voltage-dependent K⁺ channel activity.

A second possible explanation for our finding that Ca²⁺-induced relaxation is intact in the CB₁ knockout mouse, yet is blocked by SR141716, is that SR141716A is blocking a nonclassical cannabinoid receptor. Kunos and colleagues have recently demonstrated that abnormal cannabidiol, an analog of the nonpsychoactive marijuana-constituent cannabidiol, causes relaxation of the isolated rat mesenteric bed. They further found that both anandamide and abnormal cannabidiol have mesenteric vasodilator activity in the CB₁ knockout mouse, and the effect of both compounds is antagonized by SR141716A or cannabidiol. These findings have been interpreted to indicate that anandamide and other endocannabinoids can modulate vascular function by activating a nonclassical cannabinoid receptor. Of interest, in the present report, we show that O-1918, a novel analog of cannabidiol that blocks abnormal cannabidiol-induced relaxation in isolated rat and mouse mesenteric arteries (S. Batkai and G. Kunos, unpublished observations), blocks Ca²⁺-induced but not acetylcholine relaxation in isolated mouse arteries. This observation provides further support for the hypothesis that Ca²⁺-induced relaxation is mediated by sensory nerve dependent release of an endocannabinoid, possibly anandamide, which diffuses to underlying vascular smooth muscle and activates a specific anandamide receptor, which induces hyperpolarizing relaxation.

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