Am J Physiol Heart Circ Physiol
280: H1113–H1121, 2001.

Endothelium-independent, ouabain-sensitive relaxation of bovine coronary arteries by EETs

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Received 21 July 2000; accepted in final form 4 October 2000

Pratt, Phillip F., Pinlan Li, Cecilia J. Hillard, Jason Kurian, and William B. Campbell. Endothelium-independent, ouabain-sensitive relaxation of bovine coronary arteries by EETs. *Am J Physiol Heart Circ Physiol* 280: H1113–H1121, 2001.—Endothelium-derived hyperpolarizing factor (EDHF) is released in response to agonists such as ACh and bradykinin and regulates vascular smooth muscle tone. Several studies have indicated that ouabain blocks agonist-induced, endothelium-dependent hyperpolarization of smooth muscle. We have demonstrated that epoxyeicosatrienoic acids (EETs), cytochrome *P*-450 metabolites of arachidonic acid, function as EDHFs. To further test the hypothesis that EETs represent EDHFs, we have examined the effects of ouabain on the electrical and mechanical effects of 14,15- and 11,12-EET in bovine coronary arteries. These arteries are relaxed in a concentration-dependent manner to 14,15- and 11,12-EET ($EC_{50} = 6 \times 10^{-7}$ M), bradykinin ($EC_{50} = 1 \times 10^{-9}$ M), sodium nitroprusside (SNP; $EC_{50} = 2 \times 10^{-7}$ M), and bimakalim (BMK; $EC_{50} = 1 \times 10^{-7}$ M). 11,12-EET-induced relaxations were identical in vessels with and without an endothelium. Potassium chloride ($1–15 \times 10^{-3}$ M) inhibited [^{3}H]ouabain binding to smooth muscle cells but failed to relax the arteries. Ouabain (10^{-5} to 10^{-4} M) increased basal tone and inhibited the relaxations to bradykinin, 11,12-EET, and 14,15-EET, but not to SNP or BMK. Barium (3×10^{-5} M) did not alter EET-induced relaxations and ouabain plus barium was similar to ouabain alone. Resting membrane potential (E_m) of isolated smooth muscle cells was -50.2 ± 0.5 mV. Ouabain (3×10^{-5} and 1×10^{-4} M) decreased E_m (-48.4 ± 0.2 mV), whereas 11,12-EET (10^{-7} M) increased E_m (-59.2 ± 2.2 mV). Ouabain inhibited the 11,12-EET-induced increase in E_m. In cell-attached patch clamp studies, 11,12-EET significantly increased the open-state probability (NP_o) of a calcium-activated potassium channel compared with control cells (0.26 ± 0.06 vs. 0.02 ± 0.01). Ouabain did not change NP_o but blocked the 14,15-EET-induced increase in NP_o. These results indicate that: *1*) EETs relax coronary arteries in an endothelium-independent manner, *2*) unlike EETs, potassium chloride does not relax the coronary artery, and *3*) ouabain inhibits bradykinin- and EET-induced relaxations as has been reported for EDHF. These findings provide further evidence that EETs are EDHFs.

potassium channels; endothelium-derived hyperpolarizing factor; membrane potential; bimakalim; sodium nitroprusside; potassium; bradykinin

ENDOTHELIAL CELLS RELEASE soluble, transferable factors that alter the electrical and mechanical properties of adjacent vascular smooth muscle cells (20, 37, 42). ACh, bradykinin, and substance P stimulate endothelial cells to release at least three separate vasodilatory factors: *1*) prostacyclin, *2*) endothelium-derived relaxing factor (EDRF) or nitric oxide (NO) (19), and *3*) endothelium-derived hyperpolarizing factor (EDHF) (8, 16, 31). EDHF displays characteristics different from NO and prostacyclin. These include the ability of agonists to induce hyperpolarization in the presence of indomethacin and L-arginine analogs that block both cyclooxygenase and NO synthase, respectively (12, 14). Furthermore, the release of EDHF by muscarinic agonists is dependent on activation of the muscarinic M_2 receptor, whereas the release of PGI_2 and NO is coupled to M_1 receptors (29, 30). Finally, the relaxations to EDHF are more prominent in smaller diameter arteries, whereas NO relaxations are greater in larger vessels (28, 38). Recent studies suggest that EDHF is a cytochrome *P*-450 metabolite of arachidonic acid, an epoxyeicosatrienoic acid (EET), in the coronary artery (5, 26) and K^+ in hepatic arteries (15).

In addition, ouabain has been used to distinguish between NO and EDHF. With the use of a bioassay method, Feleteau and Vanhoutte (16) demonstrated that ACh, added to an endothelium-intact donor vessel, caused both vasodilation and hyperpolarization of a denuded detector vessel. However, when the denuded detector vessel was pretreated with ouabain, ACh-induced hyperpolarizations were abolished, whereas ACh-induced relaxations remained intact. In addition, incubation of both endothelium-intact donor and denuded detector vessels with ouabain resulted in elimination of both ACh-induced hyperpolarizations and relaxations. They concluded that ACh-induced hyperpolarizations were dependent on a functional Na^+-K^+-ATPase and that separate factors were responsible for mediating the hyperpolarization and relaxation induced by ACh. Ouabain also blocked ACh-induced relaxations of canine coronary artery in a perfusion/superfusion cascade bioassay (27) and blocked ADP-

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induced relaxations of canine coronary artery in a superfusion cascade by using porcine coronary artery endothelial cells as the donor of endothelium-derived factors (3). Interestingly, ouabain treatment of the denuded detector vessel in these studies had no effect on bradykinin or A-23187-induced relaxations. However, treatment of donor endothelial cells with ouabain resulted in a decrease in the bradykinin and A23187-induced relaxations. These results indicated that endothelium-dependent hyperpolarization is inhibited by ouabain; however, the mechanisms by which ouabain alters hyperpolarization were not investigated. In contrast, ouabain failed to inhibit ACh-induced hyperpolarization responses in other studies (7, 44). The reasons for these discrepancies are unclear.

We demonstrated (40) that arachidonic acid-induced relaxations of bovine coronary arteries are largely mediated by prostacyclin and cytochrome *P*-450 metabolite(s) of arachidonic acid, the EETs. We subsequently showed (5) that methacholine chloride stimulated the release of EETs, and methacholine-induced relaxations and hyperpolarizations were inhibited by cytochrome *P*-450 inhibitors and K channel blockers. EETs hyperpolarized and relaxed vascular smooth muscle and activated calcium-activated K (K_{Ca}) channels through a guanine nucleotide binding protein (5, 33). In addition, by using bioassay techniques, we and others (21, 39) have shown that bradykinin-induced hyperpolarization of coronary artery smooth muscle is dependent on an intact endothelium and is blocked by inhibitors of cytochrome *P*-450. These findings suggest that the EETs are EDHFs (5).

In hepatic arteries, the hyperpolarizations and relaxations to ACh and K^+ were inhibited by ouabain or barium and blocked by the combination of barium and ouabain (15). The responses to ACh were endothelium dependent and inhibited by the K channel inhibitors, charybdotoxin and apamin. The responses to K^+ were endothelium independent and not blocked by K channel inhibitors. ACh stimulated the release of K^+ in the subendothelial space, which was inhibited by K channel blockers. It was suggested that ACh activated endothelial K channels resulting in the release of K^+. These endothelial K^+ ions mediated the relaxation and hyperpolarization to ACh. This study suggested that K^+ is EDHF.

Because the actions of EDHF were inhibited by ouabain, one goal of the present study was to determine the effects of ouabain on bradykinin- and EET-induced relaxations of bovine coronary arteries. We also determined the effects of ouabain on the membrane potential (E_m) and K_{Ca} channel activity in bovine coronary smooth muscle cells. Because EETs have been shown to increase intracellular calcium in endothelial cells (22), we tested an alternative hypothesis that EETs act on the endothelium to release EDHF, possibly K^+. To test this hypothesis, studies were conducted in vessels with and without an intact endothelium and the responses to the EETs were compared with K^+. We demonstrated that ouabain attenuates the relaxations induced by both bradykinin and the EETs. Furthermore, we found that ouabain attenuates the hyperpolarization induced by 11,12-EET. Whereas 11,12-EET relaxed vessels identically in vessels with and without an intact endothelium, K^+ failed to relax the coronary artery. These studies provide additional support that EETs exert their action directly on the vascular smooth muscle and probably represent the biological activity described as EDHF.

MATERIALS AND METHODS

Vascular reactivity. Bovine hearts (2–4 kg) were obtained from a local abbatoir. The epicardial left anterior descending coronary artery was dissected, cleaned of adhering fat and connective tissue, and placed in a Krebs bicarbonate buffer containing (in mM) 119 NaCl, 5 KCl, 24 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 11 glucose, 0.02 EDTA, and 3.2 $CaCl_2$ (5, 40). The vessels were cut into rings, with care taken not to damage the endothelium. The rings (2 mm diameter) were suspended on a pair of stainless steel hooks in a 15-ml water-jacketed organ chamber. One hook was anchored to a steel rod and the other was attached to a force transducer (model FT-03C, Grass Instruments; Quincy, MA). Tension was recorded on a polygraph (model 7D, Grass). The organ chamber was filled with Krebs bicarbonate solution that was mixed with 95% O_2-5% CO_2 and maintained at 37°C. The vessels were challenged with repeated exposures to 2×10^{-2} M KCl and progressive increases in basal tension to determine the optimal resting tension. This tension was found to be 2 g for 2-mm diameter vessels. After the vessels equilibrated for 1.5 h, KCl (4×10^{-2} M) was added until reproducible contractions were obtained. The thromboxane-mimetic U-46619 (1×10^{-8} M) was then administered to increase basal tone to 50–80% of KCl-induced contraction. Cumulative additions of bradykinin (10^{-12} to 10^{-6} M), 11,12- or 14,15-EET (10^{-9} to 10^{-5} M), sodium nitroprusside (SNP; 10^{-9} to 10^{-5} M), bimakalim (10^{-9} to 10^{-5} M), and KCl (1 to 15×10^{-3} M) were made. Vessels were randomized to treatment groups and pretreated for 60 min with ouabain (10^{-5} to 10^{-4} M) or vehicle, precontracted with U46619 and cumulative additions of each agent was performed. For some experiments, the endothelium was removed by gently rubbing the lumen with a pair of forceps. The vessels were used once because U46619 is difficult to eliminate from the organ bath and there is some loss of contractile response with repeated exposure. We found, as did Feletou and Vanhoutte (16), that ouabain increased basal tone of the vascular preparation; however, this increase returned to baseline values within 1 h. Results are expressed as percent relaxation relative to the U46619-induced contraction with 100% relaxation representing the basal, pre-U46619 tension, which was 2 g.

[^{3}H]Ouabain binding in cultured vascular smooth muscle cells. Bovine coronary artery smooth muscle cells were cultured as previously described (4). Briefly, after enzymatic removal of endothelial cells, strips of denuded vessels were placed lumen-side down into gelatin-coated flasks with a M199 medium containing 10% FCS with L-glutamine (1%) and antibiotics (1% antibiotic-antimycotic solution). Smooth muscle cells migrated from the vessel to the flasks within 3 to 5 days. Once cell growth was established on the flasks, the vessels were then removed, and the cells were cultured in a M199 medium containing 20% FCS. The purity of smooth muscle cells was confirmed by positive immunostaining for smooth muscle cell α-actin. For [^{3}H]ouabain-binding experiments, the smooth muscle cells were grown in 12-well plates and used between *passages 3* and *6*.

[^{3}H]Ouabain-binding experiments were performed as described by Lau and co-workers (32). For saturation binding experiments, the cells were washed three times with 0.5 ml of K-free buffer (KFB) containing (in mM) 120 NaCl, 0.05 $CaCl_2$, 1.0 $MgCl_2$, 5.0 glucose, and 20 HEPES, 7.4 pH. [^{3}H]Ouabain was added in increasing concentrations (1, 2, 4, 8, 16, 32, 64, 128, and 256 nM), and 1 mM ouabain was used to determine nonspecific binding. After the cells were incubated for 1 h at 37°C, the cells were washed three times with 0.5 ml of cold KFB, lysed with 1 ml of 0.5 NaOH, and placed on an orbital shaker for 1 min, and the lysate was placed into a scintillation vial for liquid scintillation counting.

For competition experiments, 40 nM [^{3}H]ouabain was added in the presence of either KCl, ouabain, or 14,15-EET in KFB and incubated for 1 h at 37°C. After 1 h, the cells were washed and treated as described above.

Affinity constants and total receptor number were determined by fitting the data to the single site-binding equation with the use of nonlinear regression software (Prism, GraphPad; San Diego, CA). The inhibitory constant (K_i) values were determined from IC_{50} values by using the formula of Cheng and Prusoff (9).

Isolation of vascular smooth muscle cells from small bovine coronary arteries. A branch of the coronary artery was cannulated and filled with 10–20 ml of ice-cold 3% Evans blue in 50 mM sodium phosphate containing 0.9% sodium chloride, pH 7.4 PSS, and 6% albumin. The heart was dissected into 2 × 3 × 1-cm pieces and sliced into 300-μm-thick sections. Small coronary arteries stained with Evans blue were identified under a dissecting stereomicroscope. These arteries were microdissected, pooled, and stored in ice-cold PSS. The dissected coronary arteries were first incubated for 30 min at 37°C with collagenase type II (340 U/ml, Worthington), elastase (15 U/ml, Worthington), 1,4-dithiothreitol (1 mg/ml), and soybean trypsin inhibitor (1 mg/ml) in HEPES buffer consisting of (in mM) 119 NaCl, 4.7 KCl, 0.05 $CaCl_2$, 1 $MgCl_2$, 5 glucose, and 10 HEPES, pH 7.4. The digested tissue was agitated with a glass pipette to free the vascular smooth muscle cells, and the supernatant was collected. The remaining tissue was digested further with fresh enzyme solution, and the supernatant was collected at 5-min intervals for an additional 15 min. The supernatants were pooled and diluted 1:10 with HEPES buffer and stored at 4°C until used.

Potassium channel current recordings in vascular smooth muscle cells. Single-channel K_{Ca} currents were recorded by using the patch-clamp technique described by Hamil et al. (23). This K_{Ca} channel has been previously characterized in this preparation (34). For these studies, the cell-attached configuration was used to study the effects of ouabain and/or 11,12-EET on K_{Ca} currents in vascular smooth muscle cells. Patch pipettes were made from borosilicate glass capillaries pulled with the use of a two-stage micropipette puller (model PC-87, Sutter) and heat-polished by using a microforge (model MF-90, Narishige). The pipettes had tip resistances of 8–10 MΩ for single-channel recording when filled with 145 mM KCl solution. Smooth muscle cells were placed in a 1-ml perfusion chamber mounted on the stage of a Nikon inverted microscope. After the tip of the pipette was positioned on a cell, a high-resistance seal (5–15 GΩ) was formed between the pipette tip and the cell membrane by applying a light suction. The activity of K_{Ca} channel in the membrane spanning the pipette tip was recorded. These measurements represent the cell-attached mode. A patch-clamp amplifier (model EPC-7, List Biological Laboratories; Campbell, CA) was used to record single-channel currents. The amplifier output signals were filtered at 1 kHz with an eight-pole Bessel filter (Frequency Devices; Haverhill, MA). The currents were digitized at a sampling rate of 3 kHz and stored on the hard drive of a Gateway 486 DS66 computer for off-line analysis. Data acquisition and analysis were performed with pCLAMP software (version 5.7.1, Axon Instruments; Burlingame, CA). Average channel activity (NP_o) in patches were determined from recordings of several minutes by the equation

$$NP_o = \left(\sum_{j=1}^{N} t_j j \right) \Big/ T$$

where N is the maximal number of channels observed in conditions of high levels of P_o. P_o is the open-state probability, T is the duration of the recording, and t_j is the time with $j = 1, 2, \ldots N$ channels open.

Membrane potential recordings in isolated smooth muscle cells. Membrane potential was recorded with the use of the whole cell current-clamp method. Isolated smooth muscle cells were placed in a 1-ml perfusion chamber mounted on the stage of a Nikon inverted microscope. After the pipette tip was positioned on a cell, a tight seal was created, and the membrane within the pipette disrupted by applying a large pulse of suction to establish whole cell recording mode. For E_m recordings, the current-clamp mode on the EPC-7 patch-clamp amplifier was used. The E_m was monitored at the V-monitor output and continuously recorded on a polygraph (model 7D; Grass). The pipette solution contained (in mM) 145 KCl, 1 $MgCl_2$, 10 HEPES, 2 EGTA, 1 ATP, 0.5 GTP, and 300 nM ionized calcium, pH 7.2. The bath solution contained (in mM) 140 NaCl, 4.7 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 5 glucose, and 10 HEPES, pH 7.4. The effect of 11,12-EET (10^{-7} M) was tested in the presence and absence of ouabain or iberiotoxin.

Statistics. Vessels were randomly assigned to a treatment group with at least one vessel serving as a control each day. Statistical analysis was performed by an analysis of variance to determine significant differences among groups followed by Dunnett's modification of the t-test to determine differences between groups. A value of $P < 0.05$ was considered statistically significant.

Materials. All of the chemicals were purchased from Sigma Chemical (St. Louis, MO). The EETs were synthesized in our laboratory according to the method of Corey and co-workers (5, 10). Bimakalim was provided by Dr. Garrett Gross.

RESULTS

Vascular smooth muscle reactivity. Ouabain increased basal tone and this increase returned to baseline after 1 h, as previously reported (16). Pretreatment with ouabain did not significantly alter the contractions induced by U46619 (data not shown). Bradykinin relaxed the U46619-precontracted coronary arteries with an EC_{50} of 1×10^{-9} M (Fig. 1). Pretreatment with ouabain (3×10^{-5} M) resulted in attenuation of bradykinin-induced relaxations (Fig. 1*A*). Increasing the concentration of ouabain to 1×10^{-4} M provided no further attenuation of bradykinin-induced relaxations (data not shown). The 14,15- and 11,12-EET induced a concentration-dependent relaxation of U46619-precontracted vessels ($EC_{50} = 6 \times 10^{-7}$ M) (Fig. 1*B* and Fig. 2). The relaxations to 11,12-EET were identical in the presence and absence of an intact endothelium (Fig. 2, *A* and *B*). Under both conditions, pretreatment with ouabain attenuated 11,12-EET-induced relaxations. In contrast, barium (3×10^{-5} M), failed to block the relaxations to 11,12-EET. The com-

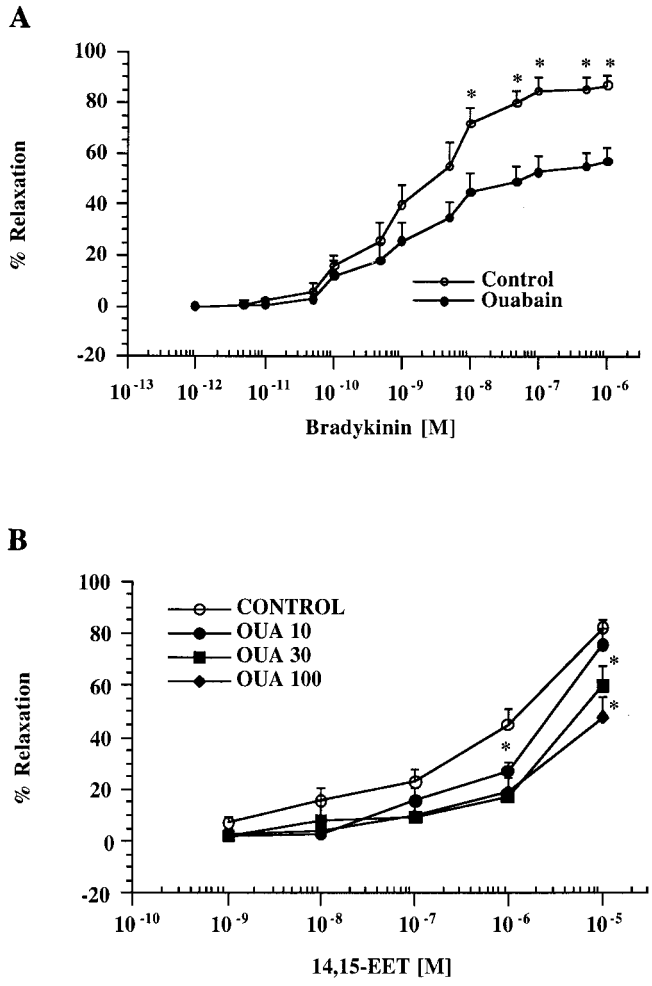

Fig. 1. Effects of ouabain (Oua) on bradykinin- (*A*) and 14,15-epoxyeicosatrienoic acids (EET)-induced relaxations (*B*) of bovine coronary arteries. Oua (3×10^{-5} and 1×10^{-4} M) was added to the organ chamber with vessels at resting tone (2 g). Tension was continuously recorded before the addition of U46619. Rings of coronary artery (1–2 mm diameter) were pretreated for 60 min with Oua, before the addition of U46619 (1×10^{-8} M). Once stable contractions were achieved, increasing concentrations of bradykinin or 14,15-EET were added to the organ chambers and maximal relaxations were recorded. Data are means ± SE; *n*, 10–12 hearts per group. *Significantly different from control, $P < 0.05$.

bination of barium and ouabain inhibited 11,12-EET-induced relaxations to a similar extent as ouabain alone. However, at the highest concentration of 11,12-EET tested (10^{-5} M), barium and ouabain were inhibited to a greater extent than ouabain. Ouabain also inhibited the relaxations to 14,15-EET (Fig. 1*B*).

To determine whether the inhibitory effects of ouabain were specific for EET-induced relaxations, we examined the effects of ouabain on bimakalim and SNP-induced relaxations. SNP, a NO donor, caused concentration-dependent relaxations of precontracted vessels ($EC_{50} = 2 \times 10^{-7}$ M) (Fig. 3*A*). The concentration-response curve to SNP was unaffected by pretreatment with either 10^{-5} or 3×10^{-5} M ouabain. However, pretreatment with 1×10^{-4} M caused a shift to the right in the concentration-response curve to nitroprusside. The potassium channel opener bimakalim relaxed precontracted vessels in a concentration-dependent manner ($EC_{50} = 1 \times 10^{-7}$ M). Pretreatment with ouabain did not significantly alter the relaxations to bimakalim (Fig. 3*B*).

Potassium chloride elicited small and highly variable relaxations at low millimolar concentrations in the presence of an intact endothelium (Fig. 4). Removal of the endothelium did not significantly alter the magnitude of relaxations elicited by potassium but greatly reduced the variability observed.

Effects of EETs on [^{3}H]ouabain binding to cultured vascular smooth muscle cells. To investigate a possible influence of EETs on the Na^+-K^+-ATPase, we examined the ability of 14,15-EET to displace the specific [^{3}H]ouabain binding of vascular smooth muscle cells. [^{3}H]Ouabain binding to cultured bovine coronary ar-

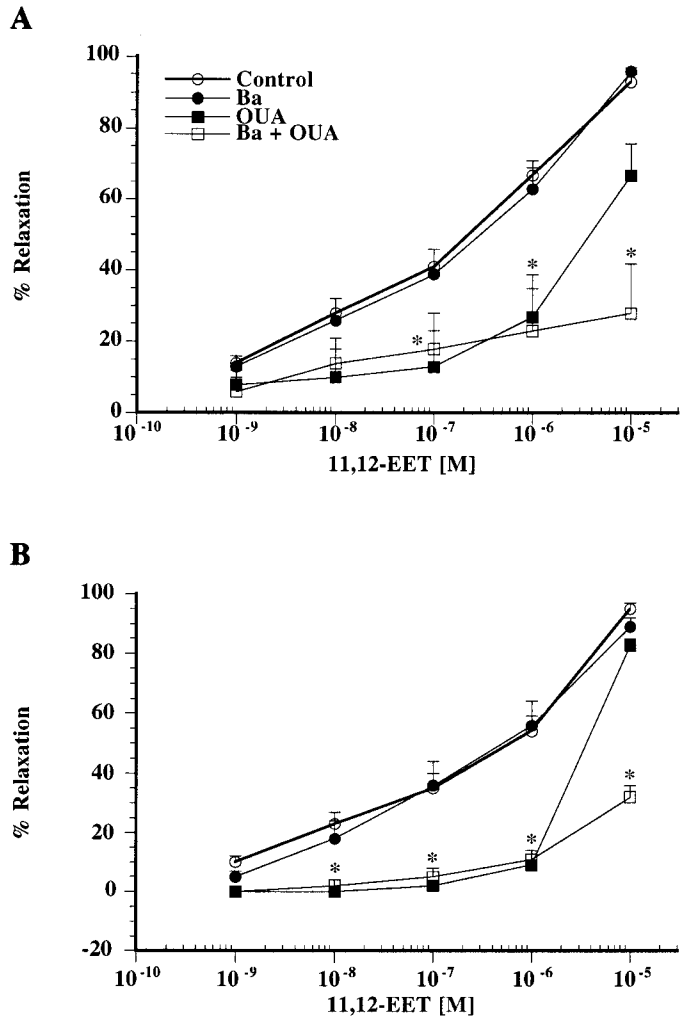

Fig. 2. Effect of Oua on 11,12-EET-induced relaxations of bovine coronary arteries. Rings of coronary artery (1- to 2-mm diameter) with (*A*) and without (*B*) an intact endothelium were pretreated for 60 min with 1×10^{-4} M Oua, 3×10^{-5} M barium (Ba) or Ba + OUA before the addition of U46619 (1×10^{-8} M). Once stable contractions were achieved, increasing concentrations of 11,12-EET were added to the organ chambers and maximal relaxations were recorded. Data are means ± SE; *n*, 4–20 hearts per group. *Significantly different from control, $P < 0.05$.

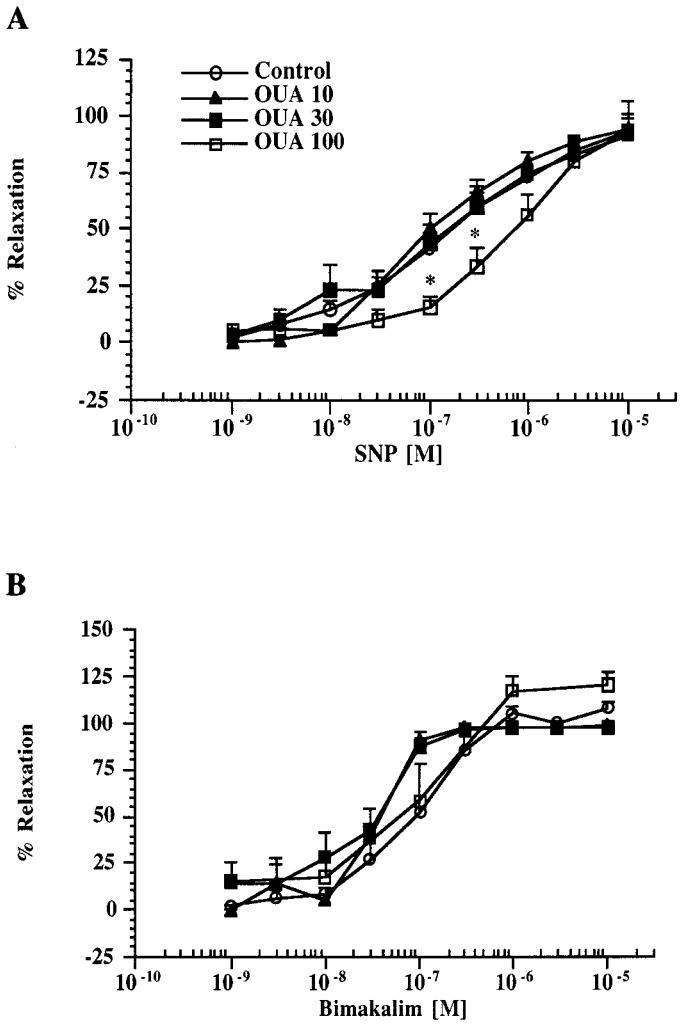

Fig. 3. Effects of Oua on sodium nitroprusside (SNP) (*A*) and on bimakalim-induced (*B*) relaxations of bovine coronary arteries. Rings of coronary artery (1–2 mm diameter) were pretreated for 60 min with the indicated micromolar concentration of Oua before the addition of U46619 (1×10^{-8} M). Once stable contractions were achieved, increasing concentrations of SNP or the potassium channel opener bimakalim was added to the organ chambers and maximal relaxations were recorded. Data are means ± SE; *n*, 5–24 hearts per group. *Significantly different from control, $P < 0.05$.

tery smooth muscle cells was specific and saturable over the concentration range of 1–150 nM (data not shown). Scatchard analysis revealed a single binding site with a dissociation constant (K_d) of 26 ± 4 nM. KCl competitively displaced [^{3}H]ouabain with a K_i of 1.0 mM, whereas 14,15-EET failed to displace [^{3}H]ouabain. These data indicate that the inhibitory effect of ouabain on EET-induced relaxation and hyperpolarization is not a result of direct competition with Na^+-K^+-ATPase at the ouabain-binding domain.

Electrophysiological studies. In isolated bovine coronary arterial smooth muscle cells, the resting E_m was −50.2 ± 0.5 mV. Addition of 11,12-EET (1×10^{-7} M) hyperpolarized the cell as indicated in the typical tracing in Fig. 5*A*. 11,12-EET-induced hyperpolarization was inhibited by pretreatment with the K_{Ca} channel inhibitor iberiotoxin (Fig. 5*B*). Ouabain (3×10^{-5} and 10^{-4} M) decreased the E_m, depolarized the smooth muscle and completely blocked 11,12-EET-induced increase in E_m (Fig. 5*C*).

Single-channel recordings of the K_{Ca} channel are shown in Fig. 6. 11,12-EET (1×10^{-7} M) resulted in a significant increase in NP_o (Fig. 6*B*). Ouabain at 10^{-5} M ($NP_o = 0.02 \pm 0.02$) and 3×10^{-5} M ($NP_o = 0.01 \pm 0.01$) had no effect on NP_o compared with control ($NP_o = 0.02 \pm 0.01$); however, 10^{-4} M ouabain increased NP_o (Fig. 6*B*). The increase with ouabain was less than that caused by 11,12-EET. When the cells were treated with ouabain (10^{-4} M), the effects of 11,12-EET were completely blocked (Fig. 6*B*). These data indicate that ouabain prevented the 11,12-EET-induced activation of K_{Ca} channels in vascular smooth muscle cells. Neither ouabain nor 11,12-EET changed the current amplitude (Fig. 6*C*).

DISCUSSION

Rosolowsky et al. (40) demonstrated that the endothelium-dependent relaxations to arachidonic acid in bovine coronary arteries were mediated by a cyclooxygenase and cytochrome *P*-450 metabolite of arachidonic acid. Further studies (41) revealed that prostacyclin and the EETs were produced by endothelial cells and were the candidates most likely responsible for the observed relaxant effects. We have recently provided evidence (5) supporting a role for the EETs as EDHFs. This conclusion was based on the findings that EETs *1*) relax precontracted bovine coronary arteries in a concentration-dependent manner, *2*) hyperpolarize perfused segments of coronary arteries, *3*) are synthesized by endothelial cells, and *4*) increase the open channel probability of the K_{Ca} channel. They are released by methacholine chloride and methacholine-induced hyperpolarization is blocked by inhibitors of cytochrome *P*-450. Whereas studies (1, 6, 26, 39) from several other

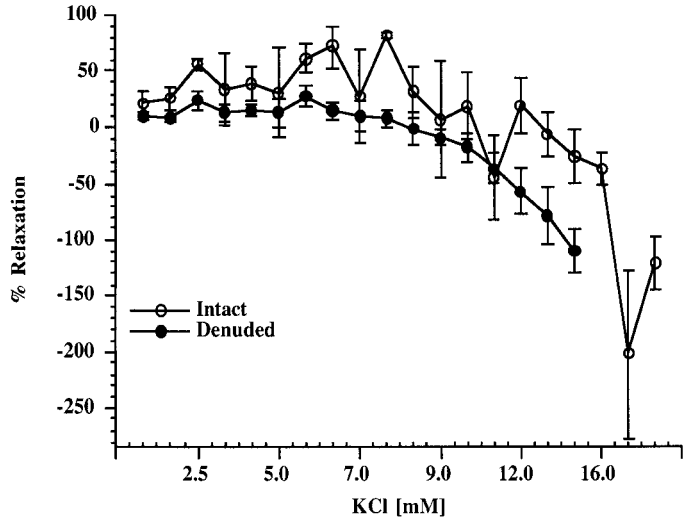

Fig. 4. KCl fails to relax U46619 precontracted bovine coronary arteries. Rings of coronary artery (with or without an intact endothelium) were precontracted with U46619 (1×10^{-8} M) before addition of KCl at the concentrations indicated. Data are means ± SE; *n*, 2–11 hearts per group.

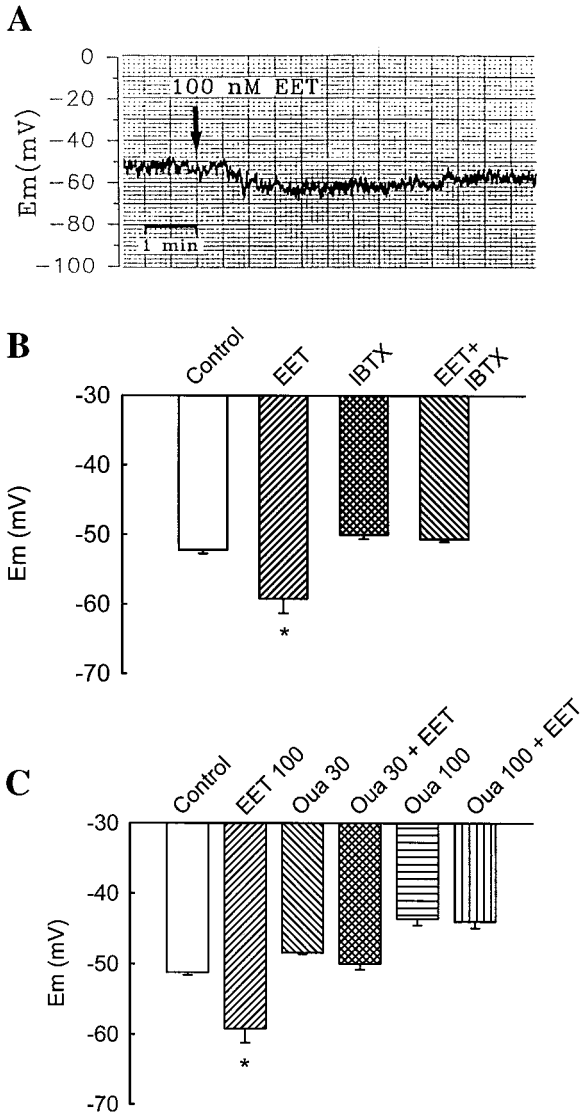

Fig. 5. 11,12-EET hyperpolarized bovine coronary arterial smooth muscle cells in an iberiotoxin (IBTX) and Oua-sensitive manner. *A*: typical tracing of change in membrane potential induced by 11,12-EET. *B*: bovine coronary artery smooth muscle cells were treated with 10^{-8} M of 11,12-EET, IBTX (100 nM) alone or in combination. *C*: bovine coronary artery smooth muscle cells were treated with 11,12-EET in the presence or absence of the indicated micromolar concentration of Oua. Data are means ± SE for change in membrane potential from 4–6 cells. *Significantly different from control, $P < 0.05$.

laboratories support this conclusion, it may not apply to all vascular beds (11, 18, 36).

Ouabain is a cardiac glycoside that blocks the relaxation and hyperpolarization caused by EDHF (16). In addition, ouabain has been demonstrated to block endothelium-dependent relaxations induced by arachidonic acid in canine coronary arteries (43). In these studies, indomethacin failed to completely block the relaxations to arachidonic acid or ACh, suggesting the existence of a factor other than a prostaglandin. Our studies in bovine coronary arteries indicate that the other factor is an EET (40). In support of this possibility, ouabain blocked the relaxations to arachidonic acid but not the relaxations to the monounsaturated fatty acids, oleic, and elaidic acids (43). The inability of these monounsaturated fatty acids to be converted to EETs may explain the lack of inhibition by ouabain.

In the present study, we found that ouabain blocks a portion of bradykinin-induced relaxations and inhibits relaxations to 14,15- and 11,12-EET in precontracted bovine coronary arteries. The inhibition was specific for bradykinin and 14,15- and 11,12-EET because ouabain did not inhibit the relaxations to the potassium channel opener bimakalim or the NO donor SNP. At the highest concentration tested, ouabain did inhibit the relaxations to SNP. Also, ouabain blocked EET-induced hyperpolarization of isolated smooth muscle cells and inhibited the EET-induced activation of K_{Ca} channels. The action of ouabain on EET-induced relaxation does not appear to directly involve the Na^+-K^+-ATPase. Smooth muscle cells cultured from bovine coronary arteries have a specific, saturable, high-affinity binding site for ouabain, the Na^+-K^+-ATPase. This binding of ouabain was not altered by addition of 14,15-EET but was inhibited by KCl. The K_d for ouabain binding was 26 nM; however, 1,000-fold higher concentrations (30–100 μM) were required to inhibit EET- and bradykinin-induced relaxations. The current and previous studies (16) do not clearly explain how ouabain blocks the action of EDHF or EETs. However, like EDHF, 11,12-EET hyperpolarizes vascular smooth muscle and activates K_{Ca} channels, and these actions of the EET and EDHF are blocked by ouabain. These data further support the view that the EETs represent EDHFs.

A recent study (15) indicates that ACh activates charybdotoxin- and apamin-sensitive K channels in endothelial cells and promotes the efflux of endothelial K^+. The K^+ activates the Na^+-K^+-ATPase and barium-sensitive K channels in smooth muscle cells causing hyperpolarization and relaxation. Potassium chloride (5–20 mM) also caused hyperpolarization and relaxation that was blocked by the combination of ouabain and barium. In endothelial cells, EETs increase intracellular calcium concentrations (22) and may activate K_{Ca} channels. This raises the possibility that EETs act on endothelial cells to release K^+, and K^+ may mediate the hyperpolarization and relaxation to the EETs. However, our results do not support this possibility for three reasons. First, the relaxations to 11,12-EET were identical in arteries with and without an intact endothelium. If the EET acted on endothelial cells to release K^+, EET would not act in vessels without an intact endothelium. Second, barium did not alter the relaxations to the EETs, and the combination of ouabain plus barium inhibited EET-induced relaxations to a similar extent as ouabain alone. Only the highest concentration of EET tested was attenuated by the addition of ouabain and barium. It is unclear whether this indicates an action on a separate type of K channel. The relaxation and hyperpolarization to KCl was partially inhibited by barium and by ouabain and completely inhibited by the combination (15). These data indicate that EETs, unlike KCl, do not affect barium-

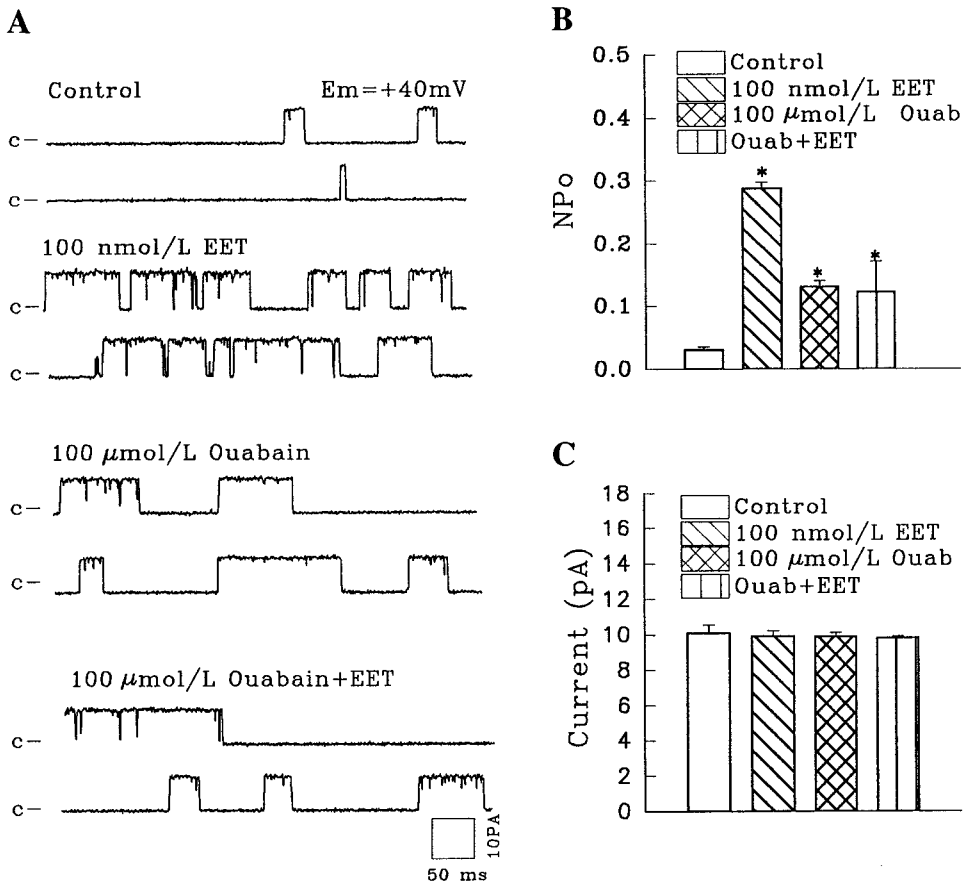

Fig. 6. Effects of Oua on EET-induced increase in K_{Ca} channel activity. *A*: single channel K_{Ca} currents were measured in cell-attached patches of isolated bovine coronary artery smooth muscle cells in the presence and absence of Oua and 14,15-EET. c, closed. *B*: summarized data from 4–6 cells on open-channel probability. *C*: summarized data from the same cells on current amplitude. *Significantly different from control, $P < 0.05$.

sensitive K channels. Third, K^+ failed to relax bovine coronary arteries if the endothelium was removed. When the endothelium was present, there was some relaxation to K^+; however, the effect was highly variable and not concentration-related. Because K^+ is thought to act by stimulating Na^+-K^+-ATPase on the smooth muscle, it should act in the absence of the endothelium. The reason for the relaxations in vessls with an intact endothelium is not clear. However, the concentrations of KCl tested appear adequate to activate the Na^+-K^+-ATPase because they inhibit ouabain binding to coronary smooth muscle cells. While the bovine coronary artery releases EDHF in response to ACh and bradykinin (21, 40), the failure of KCl to cause relaxation casts doubt on a possible role for K^+ as EDHF in this vessel. These findings support a role for EETs and not K^+ as EDHF in coronary arteries.

The physiological role for an endogenous hyperpolarizing factor remains unclear. Circulating factors, such as ANG II, antidiuretic hormone, atrial natriuretic peptide, and aldosterone are important in the long-term control of blood pressure because of their ability to alter sodium balance and ultimately affect the regulation of plasma volume. Local hormones, like NO, appear to regulate blood pressure because inhibition of its synthesis elevates blood pressure (13). Endogenous digitalis-like factor (EDLF) is another example of a substance that could regulate long-term control of blood pressure. Hamlyn et al. (24) have reported the existence of an EDLF which was isolated from human plasma. This factor blocks Na^+-K^+-ATPase activity in the same manner as ouabain. In fact, EDLF appears to be an isomer of ouabain (35). In a recent review, Blaustein (2) outlined the physiological significance of circulating ouabain, in particular, its effects on intracellular calcium concentrations in vascular smooth muscle. Inhibition of Na^+-K^+-ATPase results in an increase in intracellular sodium concentrations and hence a reduction in the electrochemical gradient for sodium. It is this gradient that drives the Na^+/Ca^{2+} exchanger, a major mechanism that removes calcium from the vascular smooth muscle cell after agonist stimulation. By reducing the activity of the Na^+/Ca^{2+} exchanger, intracellular calcium concentrations remain slightly elevated after each stimulation. The excess intracellular calcium must then be removed by action of either the plasmalemmal Ca^{2+}-Mg^{2+}-ATPase or by the Ca^{2+} pumps located on the sarcoplasmic reticulum. This extra loading of the sarcoplasmic reticulum allows more calcium to be released upon the next stimulation and thus increases vascular tone. Therefore, it is plausible that the EETs may represent natural physiological antagonists of EDLF, analogous to the ability of prostacyclin to be a physiological antagonist of the actions of thromboxane A_2. Further studies would be needed to confirm this hypothesis.

Alternatively, EDHF activity could be reduced in some forms of experimental hypertension (17, 25, 45).

If EDHF, like NO, is important in the normal regulation of vascular tone and blood pressure, inhibition of the action of EDHF by ouabain or an EDLF might elevate blood pressure and explain the hypertensive effect of EDLF.

In summary, we have reported that ouabain inhibits EET-induced relaxations of bovine coronary arteries. This effect appears to be specific in that the relaxations to bimakalim and SNP were not blocked by ouabain. Ouabain also blocked the hyperpolarizations induced by the 11,12-EET as well as EET-induced increases in K_{Ca} channel activity of isolated vascular smooth muscle cells. The relaxations to the EET are the same in the presence and the absence of the endothelium indicating that the principal action of the EETs is on the vascular smooth muscle. Unlike EET, KCl failed to relax the coronary artery. Because ouabain is known to block the actions of EDHF (3, 16, 27), these data provide further support that the EETs and not K^+ represent the activity described as EDHF in the bovine coronary artery.

The authors thank Gretchen Barg for secretarial assistance and Jennifer Trimble for technical assistance.

This work was supported by National Heart, Lung, and Blood Institute Grant HL-51055.

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