Brief Communication

Cultured Human Vascular Smooth Muscle Cells with Functional Thromboxane A2 Receptors: Measurement of U46619-Induced 45Calcium Efflux

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Thromboxane A2 (TXA2) and prostaglandin H2 (PGH2) are potent vasoconstrictors whose contractile effects are mediated by increases in cellular calcium. Stable analogues of these compounds have shown calcium ionophore activity at high concentrations. To determine if effects of TXA2/PGH2 analogues on 45Ca2+ fluxes are receptor mediated, the effects of the stable TXA2/PGH2 mimetic U46619 and the TXA2/PGH2 receptor antagonist I-PTA-OH on 45Ca2+ fluxes in cultured human vascular smooth muscle cells were studied. The smooth muscle cells were cultured from human saphenous vein explants, and they retained the morphologic and immunologic characteristics of vascular smooth muscle cells. U46619 stimulated 45Ca2+ efflux in a dose-dependent manner with an EC50 of 398 ± 26 nM (n = 4). The maximal 45Ca2+ efflux in response to U46619 (5 μM) was significantly greater (p = 0.006) than the 45Ca2+ efflux induced by KCl (40 mM). I-PTA-OH inhibited the U46619-induced 45Ca2+ efflux but had no effect on KCl-induced 45Ca2+ efflux. These results suggest that the effects of U46619 in increasing vascular smooth muscle cell calcium efflux are receptor mediated. Furthermore, vascular smooth muscle cells with functional TXA2/PGH2 receptors were cultured from human saphenous veins and provide a potentially useful in vitro system for the further study of TXA2/PGH2 receptor-mediated phenomena in human vascular tissue. (Circulation Research 1987;60:952-956)

Prostaglandin H2 (PGH2), thromboxane A2 (TXA2), and their mimetics contract vascular smooth muscle from many different species and vascular beds.1-3 These effects are thought to be mediated by stimulation of specific cellular TXA2/PGH2 receptors. However, ionophoric activity of the TXA2/PGH2 mimetic 15S-hydroxy-9α, 11α-epoxymethano)prosta-5Z,13E-dienoic acid (U44069) has been demonstrated.4,5 U44069 increases canine pulmonary vascular tone by releasing Ca2+ from an intracellular, tightly bound source.6 Similarly, U44069 activates rabbit aortic smooth muscle by liberating Ca2+ from a high-affinity intracellular pool as well as by activating a Ca2+ influx pathway.7 Use of a specific TXA2/PGH2 receptor antagonist to antagonize the TXA2 mimetic-induced 45Ca2+ efflux in vascular smooth muscle has not been reported.6 Such a study could support the idea that these effects are receptor mediated and not the result of ionophoric activity.

The availability of a pure culture of nonembryonic human vascular smooth muscle cells would allow for characterization of human vascular TXA2 receptors, in particular their structure, function, and the mechanism by which TXA2 mimetics produce their vasoconstrictor effects. In this study, vascular smooth muscle cells were cultured from human saphenous vein explants, and the effects of the TXA2 mimetic 15S-hydroxy-11α, 9α-(epoxymethano)prosta-5Z,13E-dienoic acid (U46619) and the specific TXA2/PGH2 receptor antagonist 9,11-dimethylmethano-11,12 methano-16-(3-Iodo-4-hydroxyphenyl)-13,14 dihydro-13-aza-15αβω-tetranor-TXA2 on 45Ca2+ efflux from these cells were determined (I-PTA-OH).

Materials and Methods

Dulbecco’s minimal essential medium (DMEM), trypsin-EDTA and antibiotic-antimycotic mixture (10,000 U/ml penicillin, 25 μg/ml amphotericin B, 10 mg/ml streptomycin) and fetal calf serum were purchased from Gibco, Grand Island, N.Y. Corning culture flasks and centrifuge tubes were purchased from Fisher Scientific, Pittsburgh, Penn. 45CaCl2 was purchased from Amersham, Arlington Heights, Ill.
U46619 was purchased from Upjohn Co., Kalamazoo, Mich. I-PTA-OH was synthesized as previously described. Rat tail collagen was purchased from Sigma Chemical Co., St. Louis, Mo. Human serum from coronary artery bypass procedures was deheparinized and defibrinated in the laboratory of Dr. John Lazarchick, Medical University of South Carolina, Charleston, S.C., and stored at −20°C before being sterile filtered through 0.4-μm Gelman filters and added to culture media. Phosphate buffered saline (PBS) was prepared by adding 1.2 mM NaHPO₄ to 0.85% NaCl (wt/vol) and adjusting the pH to 7.4 with hydrogen chloride.

**Culture of Vascular Smooth Muscle Cells**

Smooth muscle cells were cultured using a modification of the method of Ross. Unused portions of human saphenous veins from aorto-coronary bypass operations were briefly maintained in sterile lactated Ringer's buffer at room temperature. The vein segments were placed in sterile petri dishes under a laminar flow hood and dissected into 3-4-mm squares and placed intima side down on Corning 25-cm² culture flasks previously coated with collagen. The flasks were capped and inverted for 30 minutes, righted, and 2.5 ml of DMEM culture medium supplemented with 10% v/v human serum was added. The flasks were capped loosely and incubated at 37°C in a humidified atmosphere of 95% air-5% CO₂. The growth medium was exchanged every 48 to 72 hours. Confluent cells were passaged by washing the adherent cells twice with sterile PBS followed by the addition of 0.05% trypsin-EDTA for 5 minutes to loosen the cells from the matrix. The activity of the trypsin was halted by the addition of an equal volume of human serum. The resulting cell suspension was centrifuged at 100g for 10 minutes and distributed into new culture flasks at a 1:4 subculture ratio. All experiments were performed on cells cultured from a single patient between passage numbers 4, 9, and 12 (Figure 1).

The smooth muscle cell cultures generated from the explants displayed a doubling time of approximately 48 hours. The explanted cells displayed positive cytoplasmic staining for the presence of smooth muscle myosin (the antisem against smooth muscle myosin was a gift from Dr. Mark Willingham of the National Institutes of Health, Bethesda, Md.). Cultured fibroblasts (GM 0037 and GM 5758 obtained from the NIGMS Human Genetic Cell Repository, Camden, N.J.) served as a control and were negative for the presence of smooth muscle myosin. Ultrastructural examination of the cultured smooth muscle cells revealed abundant cytoplasmic filaments resembling myofilaments. The cultures retained the above properties at passage numbers 8 and 12 (Figure 1).

**45Ca Efflux Experiments**

45Ca efflux was measured in cultures bathed in PBS containing 1 mM CaCl₂ and 1 mM MgCl₂ (experimental buffer). Cell cultures in exponential growth phase were loaded with 45CaCl₂ (5 μCi/ml) in serum-free DMEM overnight. Uptake of 45Ca²⁺ reached equilibrium by 5 hours (data not shown). Prior to the experiments, flasks were rinsed for 18 minutes with experimental buffer. For measurement of 45Ca²⁺ efflux, 2 ml aliquots of experimental buffer were added to each 25-cm² flask of cells and exchanged with fresh buffer at two-minute intervals for 26 minutes. U46619 (50 mM) and I-PTA-OH (5 mM) stock solutions in ethanol were diluted in experimental buffer to appropriate concentrations and added to the flasks during minutes 32–38. Desaturation was commenced simultaneously in up to six 25-cm² flasks of 45Ca²⁺ labelled cells. Cell viability at the end of the washout experiments was 96 ± 0.6% in 20 μM U46619 and 99 ± 0.3% in 20 μM U46619 plus 5 μM I-PTA-OH as assessed by trypan blue exclusion (n = 4 each). Each aliquot (2 ml) of buffer was counted in a Beckman (Fullerton, Calif.) liquid scintillation spectrometer. The cells were dissolved in 1 N NaOH overnight and assayed for protein using the method of Lowry.

**Statistical Analysis**

A natural log transformation of the 45Ca²⁺ desaturation curves was used to obtain linear plots. The trapezoid rule for area under the curve (AUC) was used to measure the magnitude of 45Ca²⁺ efflux. The efflux data were subjected to an analysis of variance.
FIGURE 2. Representative experiment demonstrating the effects of maximally effective concentrations of potassium chloride and U46619 on 45Ca efflux from cultured vascular smooth muscle cells. Straight line represents a natural log transformation of the baseline slow component of 45Ca efflux. Flasks of vascular smooth muscle cells were rinsed with experimental buffer for 18 minutes beginning at time zero. Aliquots of experimental buffer were exchanged at 2-minute intervals thereafter for measurements of 45Ca2+ efflux. U46619 or potassium chloride was added at minute 32 and remained in the medium until minute 38.

(ANOVA) of repeated measures and compared to the AUC values using Fisher’s protected LSD test. Dose-response studies were analyzed using a log-logit transformation followed by a linear regression analysis from which the EC50 (the concentration required to produce a half-maximal response) was determined.

Data are expressed as mean ± SEM.

Results

Effects of KCl and U46619 on 45Ca2+ Efflux

45Ca2+ efflux from the control vascular smooth muscle cells resulted in a double exponential decay curve (Figure 1). The initial rapid component had a t1/2 of 2 minutes (k = 0.35 minutes−1). The remaining slower component of efflux had a t1/2 of 5.7 minutes (k = 0.12 minutes−1). All experimental agents were added to the buffer medium at 30 minutes, and the resulting changes in 45Ca2+ efflux represent effects on this high-affinity, slowly equilibrating pool.

U46619 (5 μM) produced an initially rapid 45Ca2+ efflux of large magnitude, which quickly tapered and returned to baseline (Figure 2). Potassium chloride (40 mM) stimulated 45Ca2+ efflux to a lesser extent (Figure 2). The maximal efflux of 45Ca2+ induced by U46619 was significantly greater (p = 0.006) than that elicited with maximally effective concentrations of potassium chloride. U46619-induced 45Ca2+ efflux from vascular smooth muscle cells was dose-dependent with an EC50 of 398 ± 26 nM (n = 4) and a maximally effective dose of 5 μM (Figure 3).

Effects of I-PTA-OH on U46619-Induced 45Ca2+ Efflux

The TXA2/PGH2 receptor antagonist I-PTA-OH antagonizes the vasoconstrictor effects of U46619 in human saphenous veins. I-PTA-OH (1 μM or 5 μM) added to the medium bathing the vascular smooth muscle cells produced no effects on 45Ca2+ efflux nor did it affect the potassium-chloride-induced increase in 45Ca2+ efflux (data not shown). When added prior to U46619, I-PTA-OH attenuated the 45Ca2+ efflux (Figure 4). I-PTA-OH (1 μM) shifted the U46619 EC50 from 398 ± 26 nM to 1,353 ± 261 nM (p < 0.05) (n = 4). I-PTA-OH (5 μM) further shifted the EC50 to 2,031 ± 57 nM (p < 0.001) (n = 4).

Discussion

The present study demonstrates TXA2/PGH2 receptor-mediated perturbations of 45Ca2+ efflux in a cultured vascular smooth muscle cell line derived from human saphenous veins. These cells demonstrated morphologic, ultrastructural, and immunologic characteristics consistent with vascular smooth muscle

FIGURE 3. Dose response relation of U46619-induced 45Ca efflux in cultured vascular smooth muscle cells. Each point represents the mean ± SEM of 4 experiments. •, U46619-induced 45Ca efflux; ○, U46619 induced 45Ca efflux in the presence of 5 μM I-PTA-OH; AUC, area under the curve (cm2 × 10).
The effects of I-PTA-OH on U46619-induced 45Ca efflux from cultured vascular smooth muscle cells. Arrows indicate the duration of exposure to U46619.

We examined the effects of U46619 on the slower component of calcium efflux from these cultured cells. This slow component of efflux is thought to represent the compartment of calcium originating from a high-affinity site within the vascular smooth muscle cell17-18 and is considered the fraction of intracellular Ca2+ in equilibrium with activator calcium ions.9 Receptor-mediated, thromboxane-induced vasoconstriction may be dependent on calcium influx through a calcium channel.17 U46619 increased the rate of 43Ca2+ efflux on the slowly equilibrating component with an EC50 of 398 nM. This value differs from the EC50 of 1.7 nM for U46619-induced contractions of human saphenous veins.13 The reason for the different values is unknown. However, our results are similar to those previously reported for rabbit aorta in which the EC50 of U44069 for 43Ca2+ efflux was 100 times higher than its EC50 for contracting the tissue.7

The effect of TXA2 mimetics on calcium flux may be secondary to an ionophore-like action.4,5,18 The TXA2/PGH2 receptor antagonist I-PTA-OH inhibited U46619-induced contraction of human saphenous veins with an IC50 of 0.36 µM.13 I-PTA-OH (1 µM and 5 µM) inhibited the U46619-induced 43Ca2+ efflux in the cultured vascular smooth muscle cells but had no inhibitory effect on potassium-chloride-induced calcium efflux and was itself devoid of activity. Recently, the thromboxane A2, antagonist 13-azaprostanoid acid was reported to inhibit thromboxane mimetic-induced calcium influx in rat aortic vascular smooth muscle cells.19 Thus, evidence suggests that thromboxane receptor-stimulated 43Ca2+ efflux and influx in vascular smooth muscle cells is probably mediated by TXA2/PGH2 receptors.

Though there is currently a great deal of interest in characterizing the nature of human TXA2/PGH2 receptors, investigations have been limited to studies in platelets. Pharmacologic studies have shown that the TXA2/PGH2 receptor in human platelets differs from the TXA2/PGH2 receptor in vascular tissue,13 but specific biochemical and biophysical studies in the human vascular TXA2/PGH2 receptor are lacking. Of particular importance, in regard to the present study, is that human saphenous vein segments are readily available at the completion of coronary artery bypass graft surgery. Thus, the culturing of these human vascular smooth muscle cells will facilitate studies of vascular TXA2/PGH2 receptors.

In summary, vascular smooth muscle cells were, for the first time, cultured from human saphenous vein explants. Evidence is presented that the cultured cells retained functional TXA2/PGH2 receptors since they demonstrated a dose-related perturbation of calcium efflux in response to the TXA2/PGH2 mimic U46619 which was blocked by the TXA2/PGH2 receptor antagonist I-PTA-OH. The availability of cultured smooth muscle cells from nonembryonic vascular tissue makes possible specific observations of vascular TXA2/PGH2 receptor and the subcellular consequences of TXA2/PGH2 stimulation in human vasculature.

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Key Words • cultured vascular smooth muscle cells • saphenous veins • TXA$_2$/PGH$_2$ receptor • $^{45}$calcium efflux
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_Circ Res._ 1987;60:952-956
doi: 10.1161/01.RES.60.6.952

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/60/6/952

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