Ouabain- and Marinobufagenin-Induced Proliferation of Human Umbilical Vein Smooth Muscle Cells and a Rat Vascular Smooth Muscle Cell Line, A7r5

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Background—We studied the growth-promoting effects of 2 sodium pump–selective cardiotonic steroids, ouabain and marinobufagenin, on cultured cells from vascular smooth muscle (VSMCs) from human umbilical vein and a rat VSMC line, A7r5.

Methods and Results—Both ouabain and marinobufagenin activated proliferation of these cells in a concentration-dependent manner, reflecting the cardiotonic steroid sensitivity of the specific α subunit contained within each cell source. The observed effective concentration ranges of both compounds was below that necessary to induce cytoplasmic ion alterations by sodium pump inhibition.

Conclusions—These data indicate that the ouabain-activated proliferative effect previously observed in canine VSMCs occurs in other VSMC sources. This growth effect seems to be initiated by drug interaction with the sodium pump, reflected by the affinity of the steroid for the pump, and is independent of altered transmembrane ionic gradients.

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Key Words: cells ■ drugs ■ muscle, smooth ■ vasculature

The role of the sodium pump, or Na⁺,K⁺-ATPase, in the catalysis of Na⁺ and K⁺ active transport across the plasma membrane of animal cells is well documented. Recent research in this area has shown that this enzyme complex may have functions distinct from those involved in active ion transport. Significant evidence has emerged indicating that the sodium pump can also function as a transmembrane signal transducing complex (for reviews, see Xie and Askari1 and Schoner2). Studies by Kometiani et al.,3 Haas et al.,4 and Liu et al5 have shown that low concentrations of the cardiotonic steroid ouabain activated rat cardiac myocyte hypertrophy and ERK1/2 phosphorylation and that these actions are totally independent of changes in intracellular Na⁺ and Ca²⁺ concentrations. In addition, we have demonstrated that 0.1 to 1.0 nmol/L ouabain induced proliferation of cultured canine vascular smooth muscle cells (VSMCs) via a signaling cascade involving Src, the epidermal growth factor receptor, and ERK1/2. The low concentrations of ouabain have also been shown to stimulate proliferation of human prostate smooth muscle cells.6 Together with the recent observations of endogenous production of these species of cardiotonic steroids,8,9 these studies suggest that they may function as autacoidal mediators of growth.

VSMC proliferation has been linked to a variety of adaptive and pathophysiological responses, and a potential endogenous regulator of this function would be of considerable interest. In addition, it has been suggested that these endogenous compounds could well be a part of the cardiovascular remodeling that occurs in hypertension.10 Thus, this study extends our previous work, because it addresses 3 critical functional issues: (1) Does the response occur in human VSMCs? (2) Do other autacoidal steroids evoke the same response? and (3) Does the response involve ligand interaction with the Na⁺,K⁺-ATPase?

To accomplish these goals, we have elected to assess 2 compounds, ouabain and marinobufagenin (MBG), whose endogenous levels have been suggested to correlate with cardiac and vascular growth parameters found in vivo.5–11

Methods

Ouabain was obtained from Sigma Chemical Co. MBG was purified as previously described.8 Unless stated otherwise, all other chemicals were from Sigma.

VSMC Cultures

Rat A7r5 VSMCs were obtained from the American Type Culture Collection (ATCC CRL 1444) in passage 11 and used in passages 13...
to 22. A7r5 cells were cultured in DMEM containing 10% FBS, 150 μg/mL penicillin, 150 μg/mL streptomycin, 300 μg/mL neomycin, 250 μg/mL gentamycin, and 100 μg/mL meropenem.

Human Umbilical Vein Smooth Muscle Cell Cultures

Umbilical cords were obtained from women with normal pregnancies after vaginal delivery. Three or 4 untraumatized segments (~15 cm each) were drained of blood, and the vein was cannulated and flushed with 30 mL of sterile 10 mmol/L glucose in PBS. The vein was filled with 30 mL medium containing 0.013% collagenase A (Roche), 1 mmol/L CaCl₂, 0.5 mmol/L MgCl₂, and 10 mmol/L glucose in PBS and incubated at room temperature for 30 minutes. The umbilical cord was drained of incubation medium, gently rubbed, and flushed with 30 mL of sterile 10 mmol/L glucose in PBS to remove endothelial cells. The vein was then filled with 37°C HBSS containing 0.08% collagenase type I (Worthington), 0.018% elastase (Roche), 0.038% trypsin inhibitor (Worthington), 0.2% BSA, 2 mmol/L CaCl₂, and 15 mmol/L HEPES, pH 7.4, and incubated at room temperature for 60 minutes. The incubation medium was collected, and the cord was gently rubbed and flushed with 30 mL of sterile 10 mmol/L glucose in PBS. The cells obtained were pelleted, resuspended in Medium 199 containing 20% FBS, 0.01% heparin, and 50 μg/mL endothelial cell growth supplement (BD Biosciences), and plated on two 100-mm culture dishes coated with gelatin. Cells were cultured in this medium until they reached confluence, which generally took ~3 weeks. Cells were then passaged at 1:6 and maintained in DMEM containing 20% FBS. For all experiments, human umbilical vein smooth muscle cells (HUVECs) were used at passage 2. When used, human endothelial cells were isolated as described above, cultured in Medium 199, and used as primary cultures.

Immunocytochemistry

HUVECs and human endothelial cells were cultured as described above, fixed in 4% paraformaldehyde, and immunostained as described previously. Anti–smooth muscle α-actin (Dako) was used at 1:1000 in blocking buffer (4% normal goat serum, 3% BSA, 0.1% Triton X-100 in PBS). Anti–smooth muscle myosin heavy chain mouse monoclonal antibodies (9A9) were used at 1:200; rabbit polyclonal anti–smooth muscle myosin heavy chain (BTI) was used at 1:150; mouse monoclonal anti-calponin (Sigma) was used at 1:5000; and affinity-purified rabbit polyclonal anti–smooth muscle 22α was used at 1:750. All antibody–antigen complexes were visualized with the Vectastain Elite ABC kit (Vector) and biotinylated secondary antibodies (1:250) provided by the manufacturer.

Assessment of Cell Proliferation

A7r5 cells and HUVECs were seeded at 10,000 and 30,000 cells per dish, respectively. Cells were grown for 1 day in 35-mm dishes. A7r5 cells and HUVECs were grown in 10% and 20% serum DMEM, respectively. The cells were switched to 5% and 10% serum DMEM, respectively, with or without cardiac glycoside, and counted after 5 days of culture with a Neubauer hemocytometer. The means of counts from 4 plates were used for each data point.

Western Blots for Detection of α-Isoforms of Na/K-ATPase in HUVECs and A7r5 Cells

Cultured HUVECs and A7r5 cells were washed in PBS and suspended in an ice-cold solution of 0.25 mol/L sucrose, 0.03 mol/L histidine, and 1 mmol/L EDTA. Each suspension was homogenized first in a Teflon/glass homogenizer and then by a Polytron homogenizer. The homogenates were centrifuged at 3000g for 15 minutes at 4°C to remove cellular debris. The supernatants were centrifuged at 115,000g for 60 minutes to obtain a crude membrane pellet. Such membrane pellets were also prepared similarly from the primary cultures of neonatal rat cardiac myocytes and from ventricular samples of adult rat heart. These cardiac preparations were used as positive controls, the predominant isoforms of the neonatal prepara-

Statistical Analysis

Data were analyzed statistically by 1-way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons. Values were considered to be statistically different at a value of *P*<0.05.

Results

Do Cardiotonic Steroids Evoke a Proliferative Response in Human VSMCs?

In light of our previous findings that ouabain stimulates proliferation of canine VSMCs, we wished to determine whether this response was unique to the dog or whether VSMCs from other species, especially humans, responded similarly. Because of their ready availability, we selected human umbilical blood vessels as a good source of VSMCs. Isolation of VSMCs from human umbilical blood vessels has been described previously. However, in the present studies, we have extended the characterization of cultured smooth muscle cells from human umbilical veins. Cells isolated and cultured as described in Methods were characterized in passage 2. Confluent cultures demonstrated a ‘hill and valley’ growth pattern and morphology typical of VSMCs. As illustrated in Figure 1, these cells were positive for the vascular smooth muscle markers α-smooth muscle actin, calponin, SM22α, and smooth muscle myosin. In contrast, endothelial cells isolated from the same vessels were negative for all 4 proteins. Thus, we have developed a procedure for the isolation and culturing of bona fide human VSMCs (HUVECs).

To determine whether ouabain had a functional (proliferative) effect on these cells similar to that on the canine cells, we treated HUVECs with ouabain for 5 days in exactly the same manner as was done with canine cells, which resulted in concentration-dependent stimulation of proliferation (Figure 2). Statistically significant increases in proliferation were observed at 0.1 nmol/L ouabain. Maximal stimulation of proliferation of HUVECs was at 1 nmol/L ouabain, a dose response similar to that observed for canine VSMCs. Higher concentrations of ouabain inhibited VSMC proliferation as it had done in canine cells. Thus, ouabain induces proliferation of VSMCs from both human and canine sources.

Is VSMC Proliferation Mediated Through Ligand Interaction With Na⁺,K⁺-ATPase?

Although the signal transduction cascades activated by ouabain are believed to be initiated by specific drug binding to the sodium pump, there is no direct evidence for this assertion. With the currently available ³H-ouabain ligands, analysis of their direct binding to the α₁ subunit of the sodium pump in either intact cells or membranes at the concentrations...
that stimulate proliferation is not possible. At the concentration at which the proliferative effect is observed, ie, 0.1 to 1.0 nmol/L, <2% of the sodium pump sites would be occupied, making it difficult to distinguish specific from nonspecific binding in standard binding studies.

Thus, we used cells of rodent origin to address this issue indirectly. This cell line (A7r5) contains only the α1 subunit (Figure 3), which is 3 orders of magnitude less sensitive to ouabain than the α1 subunit from either human or canine tissue. Furthermore, Haas et al have shown that high concentrations of ouabain activate the same pathway in these cells that we showed to be activated in canine cells. However, they did not assess proliferation, nor did they perform a dose-response curve. Therefore, we tested the growth-
promoting effect of ouabain in A7r5 cells in exactly the same manner as the human cells, and the data are also shown in Figure 2. The proliferative response of these cells is identical to that observed with human cells (as well as canine cells) except that there is a difference of 3 orders of magnitude in the ouabain concentration necessary to obtain the maximal proliferative response in rat tissue compared with both human and canine VSMCs. Most importantly, these data agree well with the 3-orders-of-magnitude difference in the affinities of the sodium pump α1 subunit for ouabain from the rat compared with the human and canine α1 subunit,16 both of which also contain only the α1 subunit (Figure 3 and Koksoy and Allen17). These findings strongly suggest that the proliferative actions of ouabain on VSMCs are mediated by ouabain binding to the α1 subunit of the sodium pump at concentrations that have generally been shown not to alter cytoplasmic ion levels.

Do Other Endogenous Cardiotonic Steroids Evoke a Similar Response?

To date, ouabain has been the only cardiotonic steroid tested for its ability to activate a signal transduction cascade and stimulate VSMC proliferation.3-6 In light of the evidence that the cardiotonic steroid aglycone MBG as well as ouabain may be produced endogenously in mammals,2,8,9,11 which, as indicated in the introduction, is the rationale for using these 2 compounds, we sought to determine the effects of MBG on human and rat VSMC proliferation. As observed with ouabain, MBG induced concentration-dependent stimulation of proliferation in both HUVSMCs and A7r5 cells (Figure 4), with a significant difference in the dose-response curves reflecting the sensitivity differences between the specific resident α1 subunits. Statistically significant increases in proliferation were observed at 0.1 and 10 nmol/L MBG in HUVSMCs and A7r5 cells, respectively. Maximal stimulation was observed at 1 nmol/L MBG in HUVSMCs and at 0.1 μmol/L MBG in A7r5 cells. Higher concentrations of MBG inhibited proliferation in both VSMC types (Figure 4). Once again, VSMCs from the “sensitive” human responded at lower concentrations of MBG than cells from the “nonsensitive” rat. The 2-order-of-magnitude difference in response to MBG between the human and rat cells is consistent with the hypothesis that these drugs both activate the proliferative pathway by binding to the α1 subunit of the sodium pump.

Figure 2. Effects of ouabain on proliferation of canine, human, and rat VSMCs. Rat A7r5 cells and HUVSMCs were grown as described in Methods in presence and absence of indicated concentrations of ouabain for 5 days. Cells were counted on day 5 of culture with a Neubauer hemocytometer. Data for canine VSMCs (CVSMCs) comes from Reference 6 (n=3, determined in quadruplicate). C, control; *P<0.05 vs control; #P<0.05 vs 1 nmol/L; ##P<0.05 vs 1 μmol/L.

Discussion

The purpose of these experiments was 3-fold: (1) to ascertain whether cardioactive steroids evoke a proliferative response in human VSMCs similar to that already observed in canine VSMCs; (2) to determine indirectly whether the observed VSMC proliferation is mediated through ligand interaction with the α1 subunit of the Na+,K+-ATPase; and (3) to determine whether at least 1 other putative endogenous digitalis-like factors also evokes a similar response in human VSMCs.

We have successfully isolated human VSMCs from umbilical vein (Figure 1) and have demonstrated that both ouabain and MBG can activate proliferation of these cells in the same manner as we have shown earlier for canine cells.6 Although the need for assessing this phenomenon in human cells is obvious, we recognize that the applicability of these data, obtained with such a selective source of human VSMCs, to other human cells may be limited. However, it can be said that in general, arterial cells and venous cells from mammalian species respond similarly to most pharmacological agents. It was most important to be certain that the cells being used to verify the proliferative effects of these 2 cardiotonic
steroids were in fact bona fide VSMCs, as demonstrated by the immunocytochemistry shown in Figure 1.

The proliferative response of HUVSMCs to ouabain and MBG occurred at the same concentrations, which is reminiscent of the similar concentration requirements for these agents to inhibit Na⁺/K⁺-ATPase activity in human mesenteric artery sarcolemma.¹⁸ However, inhibition of the enzyme activity occurred at 100-fold higher concentrations than that which stimulated proliferation. In contrast, A7r5 cells were 10-fold more sensitive to MBG than to ouabain. This 10-fold difference in sensitivity in rat A7r5 cells is in general agreement with the 20-fold higher IC₅₀ values for ouabain that inhibit rat aortic Na⁺/K⁺-ATPase compared with MBG.¹⁸,¹⁹ Thus, the similar rank order for ouabain- and MBG-induced proliferation and inhibition of Na⁺/K⁺-ATPase activity in human and rat VSMCs also suggests that the proliferative actions of ouabain and MBG are a result of their binding to the α-subunit of the sodium-pump.

It is also important to note that in the 3 VSMC sources used, the α₁ subunit of the sodium pump was the only readily observed isoform (Figure 3), thus allowing us to use its well-documented affinity differences between rat and other mammalian species to suggest a specific interaction of ouabain and MBG with the subunit.

A striking consistency between all of the VSMC data as well as the data from human prostate smooth muscle cells⁷ is the biphasic nature of the ouabain/MBG effect. Thus, at low concentrations, proliferation was induced, reached a plateau, and then diminished at higher concentrations. Indeed, with higher concentrations of ouabain, proliferation was induced, reached a plateau, and then diminished at higher concentrations. Indeed, with higher concentrations of ouabain, cell proliferation was diminished. The similar pharmacological profile by which these compounds stimulate proliferation and inhibit Na⁺/K⁺-ATPase activity in human and rat VSMCs as described in the literature¹⁸,¹⁹ strongly suggests that the proliferative actions of these compounds are a result of their binding to the α-subunit of the sodium pump. The suggestion has been made that for the prostate cells, higher concentrations of ouabain activate apoptosis in these cells, and although there is some evidence that this may occur in rat VSMCs as well, this is still controversial.²⁰ There is also a significant body of evidence suggesting that the growth-inhibitory effects observed in this article are quite similar to those observed with bufadienolides by other workers.²¹,²² However, it may well be that higher drug concentrations in this case simply inhibit enough pump sites to sufficiently alter cytoplasmic ion content to interfere with the activated transduction pathway. The mechanism of this inhibitory effect is currently under investigation.

In summary, the data presented here extend our earlier proliferation data obtained in canine VSMCs⁶ to rat and human VSMCs. In addition, the use of both MBG and ouabain also suggests that these drugs activate proliferative pathways by binding to the sodium pump at respective concentrations considered to be below those that would perturb cytoplasmic ionic content. It is quite likely that cardiotonic steroids have functional vascular effects in addition to their inhibitory effects on ion transport.

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References


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