The hypothesis of this study is that the sodium pump complex acts as an intracellular signal-transducing molecule in canine vascular smooth muscle cells through its interaction with other membrane and cytoskeletal proteins. We have demonstrated that 1 nM ouabain induced transactivation of the epidermal growth factor receptor (EGFR), resulting in increased proliferation and bromodeoxyuridine (BrdUrd) uptake. Immunoprecipitation and Western blotting showed that the EGFR and Src were phosphorylated within 5 min of 10^-8 M ouabain stimulation. Both ouabain-induced DNA synthesis (BrdUrd uptake) and MAPK42/44 phosphorylation were inhibited by the Src inhibitor PP2, the EGFR kinase (BrdUrd uptake) and MAPK42/44 phosphorylation were stimulated with 10^-4 M ouabain. Thus, low concentrations of ouabain, which do not inhibit the sodium pump sufficiently to perturb the resting cellular ionic milieu, initiate a transactivation signaling cascade leading to vascular smooth muscle cell proliferation.

The role of the sodium pump as a regulator of intracellular ionic balance has been well documented. However, research in this area has recently shown that this protein complex has the potential to function in ways that apparently do not involve these well documented ionic shifts. For example, Peng et al. (1) reported that the sodium pump complex can function as a molecular signal transducer in rat cardiac myocytes. Kometiani et al. (2) further showed that 10^-4 - 10^-5 M (10-100 μM) ouabain activated cardiac myocyte hypertrophy and MAPK42/44 phosphorylation. Recently many growth factor signaling pathways have been shown to involve EGFR transactivation in VSMCs (3, 4) as was also shown earlier in cardiac myocytes (5). In this paper we describe the ability of low concentrations of ouabain (0.1-1.0 mM) to induce proliferation of cultured canine vascular smooth muscle cells via a signaling cascade involving Src, EGFR, and MAPK42/44. The isolation of ouabain-like substances from the plasma and urine samples of both healthy and hypertensive individuals (6), as well as a variety of animal tissues, has suggested potentially important paradigms for these agents in the modulation of cell function through interaction with the sodium pump.

MATERIALS AND METHODS

All chemicals were obtained from Sigma. Ouabain (o-3125 and o-5754) from Sigma and ouabain (75640) from Fluka were used. All kinase inhibitors were from Calbiochem. Antibodies for Src, phospho-tyrosine (4G10, monoclonal), and EGFR were from Upstate Biotechnology Inc. (Waltham, MA). The antibody for MAPK42/44 (E10, monoclonal) was from Cell Signaling Technology Inc. (New England Biolabs, Beverly, MA). Anti-active EGFR antibody was purchased from Transduction Laboratories.

Vascular Smooth Muscle Cell Culture—Vascular smooth muscle cells were isolated from the saphenous veins of mongrel dogs by a two-step enzymatic digestion procedure as described earlier (7). The collected cells were cultured in 10% serum DMEM containing 150 μg/ml penicillin, 150 μg/ml streptomycin, 500 μg/ml neomycin, 250 μg/ml gentamycin, and 0.1 mg/ml meropeen. Upon reaching confluence the cells were passaged twice. The second passage cells were used. All cells were synchronized in serum-free media for 48 h prior to experimentation.

BrdUrd Uptake for Assessment of DNA Synthesis—The bromodeoxyuridine (BrdUrd) uptake kit from Calbiochem/Oncogene was used. This method uses spectrophotometric absorbance for the detection of BrdUrd incorporated into the DNA, visualized by using anti-BrdUrd antibodies and horseradish peroxidase-conjugated secondary antibodies. For this assay cells were cultured in 96-well plates at a concentration of 2000 cells/well. Cells were grown in serum for 48 h and synchronized for 48 h before the experiment. BrdUrd uptake during 24 h was measured. A mean of six wells was used to determine each data point.

Cytometry to Assess Cell Proliferation—Cells were grown in 35-mm dishes in 10% serum DMEM for 1 day, counted, switched to 5% serum DMEM with or without ouabain, and counted after 5 days of culture using a Neubauer hemocytometer. The mean of counts from four plates was used for each data point.

Western Blots—Canine VSMCs were grown in 10% serum DMEM in 100-mm dishes to 70% confluence, upon which they were switched to serum-free DMEM for 48 h for quiescence. For the experiments cells were stimulated with 10^-9 M ouabain in serum-free DMEM for the indicated times. After treatment with ouabain, cells were collected by a rubber scraper on ice by using sample buffer (100 μl/100-mm dish) (62.5 mM Tris, 2% SDS, 10% glycerol, 10 μg/ml aprotinin, 1 mM phenylmethysulfonyl fluoride, 10 μg/ml leupeptin, 1 mM sodium pyrophosphate, 1 mM ortho- vanadate, pH 6.8) and briefly (15 s) sonicated on ice. The samples were run on 7.5 or 10% SDS-polyacrylamide gels in Laemmli buffer, and protein bands were transferred to Immobilon-P (Millipore, Bedford, MA) nylon membranes. The gels were transferred for 1 or 5 h for optimum transfer and quantitation of small or large size bands, respectively. Membranes were incubated with either phospho-MAPK antibody (E10, monoclonal), MAPK antibodies (New England Biolabs), phospho-tyrosine (4G10) antibody, or EGFR antibody (Upstate Biotechnology Inc.) in blocking solution (5% nonfat dry milk in 1× TBST (0.1% Tween 20, 2.5% Tris base, 8% NaCl, pH 7.2)) overnight at 4°C. The proteins were detected by using goat anti-mouse or goat anti-rabbit secondary antibodies (Bio-Rad) and the ECL system (Amersham Pharmacia Biotech). The blots probed with phospho antibodies were stripped.
and reprobed with a non-phospho antibody to assure equal loading. The density of resulting protein bands was analyzed by using Image Tool (University of Texas, San Antonio, TX) or Scion Image (Scion Corp., Frederick, MD) software.

**Immunoprecipitations**—Cells were isolated as stated for Western blotting and lysed in immunoprecipitation buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 25 mM β-glycerophosphate). 200 μg of protein were precipitated in immunoprecipitation buffer, in a total volume of 1 ml, by using the antibody (5 μg/sample) and protein A/G-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h to overnight at 4 °C on a rotating platform. The beads were collected by pulse centrifuging at 10,000 × g. Beads were washed one time in immunoprecipitation buffer and three times in phosphate-buffered saline (PBS) for 20 min each. The beads were boiled in 2% SDS sample buffer for 5 min and centrifuged for 1 min at 10,000 × g to collect the immunoprecipitated protein. At this point, the protein was subjected to Western blotting as described above.

**Rubidium Uptake to Assess Na+/K+ Pump Activity**—Uptake of 86Rb+ is used as an indicator of pump function. Cells were cultured to about 70% confluence and synchronized as described above. Before the experiments, cells were washed twice with buffer consisting of 120 mM NaCl, 5 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 20 mM HEPES adjusted to pH 7.4. The cells were preincubated in the buffer for 20 min. Cells in treatment groups were incubated for 10 min in 1 nM or 0.1 mM ouabain; following this, rubidium uptake was initiated by addition of 2 μCi of 86Rb with or without 1 nM or 0.1 mM ouabain. After incubation in 86Rb+ for 10 min, the experiment was terminated by aspiration of the incubation fluid followed by four rapid washes in cold buffer. Cells were trypsinized, and a small aliquot was used to count cell numbers. Reduction in the total uptake was measured for each group and was normalized to the cell number. A duplicate set of samples was run simultaneously to determine ouabain-sensitive 86Rb+ uptake or that which is due to the Na+/K+ pump. This is calculated by subtracting the uptake in the presence of ouabain (ouabain-insensitive uptake) from total uptake of 86Rb+.

**Measurement of Intracellular Ca2+**—Intracellular Ca2+ was measured by fura-2/AM. Cells were grown in 10% serum DMEM on glass coverslips until they reached 50% confluence. At this point they were made quiescent by incubating in serum-free media. At the time of experiments, cells were washed with PBS twice and incubated for 1 h at room temperature in the presence of 10 μM fura-2/AM. At the end of this period cells were washed twice with PBS to get rid of extracellular fura-2 and were further incubated in PBS for 20 min at room temperature. After this, cells were treated with 10−9 or 10−7 M ouabain or other agents (platelet-derived growth factor and ionomycin) and intracellular calcium was measured by using the Incyt MicroPhotometer Module from Intracellular Imaging Inc. (Cincinnati, OH). The measurements were made from individual cells, and a mean of 16 cells was used.

**Statistical Analysis**—We used one-way analysis of variance followed by Tukey’s honestly significant difference test to analyze the differences within groups and Student’s t test to test differences among groups.

## RESULTS

**Ouabain Induced DNA Synthesis and Proliferation in VSMCs in a Dose-dependent Manner**—We investigated the effects of ouabain on VSMC proliferation. The control cells were treated with DMEM with or without 5% serum. Addition of 10−3 M ouabain increased BrdUrd uptake by 32% both in the presence and absence of serum compared with control in the presence of 10−9 (−8), 10−8 (−9), and 10−7 (−10) μM ouabain with or without 5% serum as measured by a kit that detects BrdUrd (BrdU) incorporated in the cells by using anti-BrdUrd antibodies and spectrophotometric absorbance. Cells were grown in 96-well plates in 10% serum DMEM for 48 h and were synchronized prior to the experiment. BrdUrd uptake during 24 h of ouabain treatment was measured (n = 8). 10−8 M ouabain induced an increase in BrdUrd uptake in the presence or absence of serum compared with appropriate control cells (C) not treated with ouabain (*, p < 0.001), b, proliferation induced by ouabain. Cells were seeded at 50,000/ dish, grown in 35-mm dishes in 10% serum DMEM for 1 day, counted, and changed to 5% serum DMEM with or without the indicated concentrations of ouabain for 5 days. The cells were counted on the 5th day of culture by using a Neubauer hemocytometer (n = 4, determined in quadruplicate). *, p < 0.05 compared with 0 nM; **, p < 0.05 compared with 0.1 nM; ##, p < 0.01 compared with 1 nM; ###, p < 0.01 compared with 0 and 5 nM.

**Figure 1**. a, DNA synthesis induced by ouabain. BrdUrd uptake of canine saphenous VSMCs in the presence of 10−9 (−8), 10−8 (−9), and 10−7 (−10) μM ouabain with or without 5% serum as measured by a kit that detects BrdUrd (BrdU) incorporated in the cells by using anti-BrdUrd antibodies and spectrophotometric absorbance. Cells were grown in 96-well plates in 10% serum DMEM for 48 h and were synchronized prior to the experiment. BrdUrd uptake during 24 h of ouabain treatment was measured (n = 8). 10−8 M ouabain induced an increase in BrdUrd uptake in the presence or absence of serum compared with appropriate control cells (C) not treated with ouabain (*, p < 0.001), b, proliferation induced by ouabain. Cells were seeded at 50,000/ dish, grown in 35-mm dishes in 10% serum DMEM for 1 day, counted, and changed to 5% serum DMEM with or without the indicated concentrations of ouabain for 5 days. The cells were counted on the 5th day of culture by using a Neubauer hemocytometer (n = 4, determined in quadruplicate). *, p < 0.05 compared with 0 nM; **, p < 0.05 compared with 0.1 nM; ##, p < 0.01 compared with 1 nM; ###, p < 0.01 compared with 0 and 5 nM.

**Ouabain Activates Tyrosine Phosphorylation**—In samples of VSMCs treated with 10−9 M ouabain, tyrosine phosphorylation of many proteins of different sizes was observed by Western blot. The tyrosine phosphorylation was maximal at 5 min (Fig. 2a). In samples transferred for 1 h, the most prominent phosphorylated bands appeared at 130, 85, 70, and 44 kDa. The proteins larger than 140 kDa could be observed only after transferring for 5 h, which affected the visibility of the smaller bands negatively. Thus in these experiments a positive control of EGF-treated cells was used to test for the effect of EGF and show the EGFR phosphorylation in canine VSMCs. In the samples transferred for 5 h, the presence of a 180-kDa band...
that was also phosphorylated by EGF was observed (Fig. 2b).

These data together suggested that ouabain induced either tyrosine kinase activation or tyrosine phosphatase inhibition while activating the signaling cascade in canine VSMCs. The signaling involved phosphorylation of many proteins, one of which seems to be the EGF receptor.

**Ouabain Activates MAPK42/44 Activation in a Concentration-dependent Manner**—We first tested the possibility that the 44-kDa protein referred to above was MAPK42/44 since MAPK42/44 activation is often induced in proliferating VSMCs (8). Indeed we observed that $10^{-9}$ M ouabain induces MAPK42/44 activation at both 5 and 15 min in the absence of serum (Fig. 3a) (*, $p < 0.001$). In addition, there was a small amount of activation observed with $10^{-8}$ M ouabain as well, but as seen in Fig. 1 there was never any increased proliferation or BrdUrd uptake observed at this concentration of ouabain. This suggests that at higher concentrations other consequences of ouabain-pump interaction, i.e. sufficient pump inhibition to lead to cellular ionic perturbations, may be interfering with the proliferative signaling pathway.

**The Effect of Inhibitors of MEK, Tyrosine Kinases, Src, and...**
EGFR on VSMC Proliferative Parameters—MEK inhibitor PD98059 (100 μM), tyrosine kinase inhibitor genistein (100 μM), the Src kinase inhibitor PP2 (500 nM), and the EGFR inhibitor AG1478 (500 nM) all inhibited both the MAPK42/44 activation (Fig. 4a) (+, p < 0.001) and DNA synthesis (Fig. 4b) (+, p < 0.002) induced by 10−9 M ouabain. DNA synthesis was not inhibited by the protein kinase C inhibitor calphostin (100 μM).

Investigation of the Role of Sodium Pump Function—We wanted to examine the possibility that sodium pump inhibition per se could be a signal inducer even at these low concentrations of ouabain (0.1–1.0 nM). We used the 86Rb uptake method to assess sodium pump activity during a 15-min 10−9 M ouabain treatment. There was no detectable change in 86Rb uptake between control and ouabain-treated cells. High concentrations of ouabain inhibited the sodium pump and decreased 86Rb uptake as expected (Fig. 5a).

We also tested our cells for the effect of a range of low K+ concentrations that would inhibit the sodium pump and thus increase intracellular sodium. Cells were grown in 96-well plates in 10% serum DMEM for 48 h and synchronized in serum-free DMEM for 48 h as usual. At this point the medium was switched to serum-free low K+ DMEM. Control cells were treated with regular serum-free DMEM, and ouabain was administered in regular DMEM as well. BrdUrd uptake of cells treated with 1–4.5 mM K+ (in 0.5 mM increments) did not increase during the 24-h stimulation compared with control. However, 10−9 M ouabain induced a 30% increase (*, p < 0.001) (Fig. 5b). These data imply that the observed signaling is not due to inhibition of sodium pump activity.

Intracellular Calcium Measurements in Ouabain-stimulated VSMCs—The importance of calcium signaling for MAPK42/44 activation and proliferation is well documented for VSMCs (9, 10). To determine whether this is a part of the ouabain signaling pathway, we measured intracellular calcium by fura-2 during ouabain treatment for 15 min in the absence of serum. 10−9 M ouabain did not induce any changes in intracellular calcium levels (Fig. 6), which suggested that at least the early steps of the signaling pathway do not involve calcium. In addition, a much higher ouabain concentration had no effect on cytoplasmic Ca2+ as well, consistent with the observation that the Na+/Ca2+ exchanger has only a minimal regulatory role in this cell type (canine VSMCs) (11). For controls, calcium was increased by both 20 ng of platelet-derived growth factor (12) (Fig. 6) and ionomycin (data not shown).

Detection of Src and EGFR Phosphorylation—In our experiments 10−9 M ouabain doubled the EGFR phosphorylation (shown by immunoprecipitations followed by blotting with anti-phosphotyrosine antibodies) at 5 min of stimulation (*, p < 0.001). The direct blots with anti-phosphotyrosine antibodies also showed a phosphorylated band at ~180 kDa (Fig. 2b). We verified the 180-kDa band to be EGFR by immunoprecipitation and immunoblotting (Fig. 7). Both EGFR- and ouabain-induced EGFR phosphorylation were blocked by AG1478 tyrosphostin treatment, which inhibits the EGFR kinase. The ouabain-induced activation of the EGFR was totally blocked by the Src inhibitor PP2, which suggests a role of Src kinase in ouabain-induced EGFR transactivation. On the other hand, EGFR-induced activation of the EGFR was not affected by PP2 (Fig. 8a) (+, p < 0.001). We were also able to show an increase in Src phosphorylation by 5 min of 1 mM ouabain stimulation in canine VSMCs (*, p < 0.05) by immunoprecipitation and immunoblotting (Fig. 8b). These data suggest that Src activation appears to be involved in the EGFR activation in ouabain-induced signaling in VSMC proliferation.
Little attention has been paid to the regulatory role that the sodium pump complex may have that is unrelated to its ionic regulatory functions, although several papers suggest a regulatory role for ouabain in molecular signaling (1, 5, 13, 14).

DISCUSSION

Recently Liu et al. (14) have shown that 100 μM ouabain can activate hypertrophic pathways in rat cardiomyocytes. The present paper defines the proliferation pathways of canine VSMCs induced by low concentrations of ouabain.

We observed at 10⁻¹⁰ and 10⁻⁹ M that ouabain induced MAPK42/44 activation, DNA synthesis, and proliferation in VSMCs. The increase in DNA synthesis and proliferation effects disappeared with increased ouabain concentrations, but a small increment in MAPK activation remained. Considering the limited number of pump sites and expression of only one isoform of the sodium pump in these cells (15), this finding suggests that at higher concentrations the pump-inhibitory effect of ouabain might interfere with its proliferative effect.

The concentrations at which we observed a significant increase in BrdUrd uptake, proliferation, and MAPK42/44 activation, DNA synthesis, and proliferation in VSMCs induced by low concentrations of ouabain were measured for 15 min. The change in fluorescence inside the cells was monitored and measured by using the InCyt MicroPhotometer Module, which consists of an inverted epifluorescence microscope, low light level integrating CCD camera, computer-controlled filter changer, Xenon UV/visible arc lamp, image-processing computer, and data acquisition/analysis software. The trace shows a mean of 16 individual recordings from different cells (n = 5). The change in [Ca²⁺]i, is the increase above resting levels, which averaged about 85 μM.

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Our data also demonstrated that MAPK42/44 activation is required for ouabain-induced DNA synthesis and proliferation and that this effect was inhibited by PD98059 (MEK inhibitor). The importance of MAPK42/44 activation for ouabain signaling is also supported by the fact that other inhibitors (PP2, AG1478 tyrphostin, and genistein) inhibit DNA synthesis in response to ouabain and also inhibit MAPK42/44 activation.
10^{-9} \text{M} \text{ouabain} \text{had no effect on}^{86}\text{Rb}^+ \text{uptake}, \text{an accepted index of Na}^+ \text{pump activity. This suggested that even if there may be a small number of pumps inhibited at this low concentration of ouabain such inhibition does not significantly affect intracellular sodium homeostasis and suggests that the signal-
ing is not due to increased intracellular Na}^+ \text{itself. Certainly higher concentrations of ouabain can increase intracellular sodium and limit proliferation. We have also measured intracellular calcium to further assess the possible effect of 10^{-9} \text{M} \text{ouabain} \text{and observed no changes, although the cells did re-
spond positively to platelet-derived growth factor (16).}

In our study, the principal pathway that was activated by ouabain was the EGFR pathway. The fact that MAPK42/44 activation did not increase further from what is observed with EGF alone when 10 ng of EGF and 10^{-9} \text{M} \text{ouabain} \text{were administered together (data not shown) suggested that EGFR transactivation is a step in the molecular cascade induced by ouabain. There is increasing evidence from a number of recent studies suggesting that transactivation of the EGFR by growth factors that use receptor tyrosine kinases as well as G-protein-
coupled receptors occurs readily in VSMCs (17–19). In our study both Src and EGFR were phosphorylated by 1 \text{nM} \text{ouab}ain. EGFR phosphorylation was blocked by Src inhibitors in cells stimulated by ouabain but not in those stimulated by EGF, which suggested a role for Src kinase in ouabain-induced EGFR transactivation.

The role of EGFR transactivation has been shown for a number of other agents, such as insulin, insulin-like growth factor-I, \alpha_{1c}-adrenergic receptor agonists, and angiotensin II (20–23). This is the first time that this pathway has been shown to be involved in ouabain-induced proliferation in VSMCs, although it has been shown to be involved in cardiomyocyte hypertrophy (5). The fact that low concentrations of K^+ (1–4.5 mM), which are known to inhibit the pump at least partially, did not induce the signaling pathways induced by ouabain provides important evidence for a unique signaling mechanism that is not due to pump inhibition but rather receptor-ligand binding (pump-ouabain) in VSMCs. EGFR trans-
activation seen by ouabain treatment is intriguing because previously EGF has been shown to induce sodium pump expression and thus reduce lung edema in pulmonary epithelial cells (24). This suggests a two-way interaction/activation between two signaling systems. The cytoskeletal proteins ankyrin and adducin (25, 26) and cytoskeleton-related kinases such as focal adhesion kinase are good candidates for mediat-
ing the link between a conformational change in the sodium pump to its neighboring proteins in the membrane (27).

Ouabain-induced signaling is intriguing considering that recently many researchers have isolated a variety of substances commonly termed endogenous digitalis-like factors from plasma or urine of healthy as well as hypertensive individuals (28–31). Similar substances have also been isolated from ex-

**FIG. 7. EGFR activation.** Cells were grown and prepared as de-
scribed for Western blotting and stimulated for 5 min with 1 \text{nM} \text{ouab}ain. Samples were lysed and immunoprecipitated in immunoprecipi-
tation buffer with 4 \mu g of EGFR antibody and protein A/G-Sepharose beads. The immunoprecipitates were subjected to 7% SDS-polyacryl-

**FIG. 8. a, inhibitors of EGFR phosphorylation.** Synchronized cells were preincubated with inhibitor alone for 20 min before being stimu-
lated with 1 \text{nM} \text{ouabain} or 10 ng of EGF in the presence of an inhibitor. Src inhibitor PP2 (500 \text{nm}) and EGFR inhibitor AG1478 (500 \text{nm}) were used. Experiments were carried out in the absence of serum. PP2

- **Control**: E, ouabain; 4, ouabain; 0, ouabain + AG1478 tyrphostin; \(E + AG\), ouabain + AG1478 tyrphostin; minutes.
- **5' ouabain**: C, control; ouab; ouabain; E, EGF; E + O, EGF + ouabain; E + P2, EGF + PP2; O + AG, ouabain + AG1478 tyrphostin; \(E + AG\), EGF + AG1478 tyrphostin; minutes.
Vascular Smooth Muscle Proliferation Is Induced by Ouabain

In summary the study presented here suggests a potential new role for the sodium pump in signaling, one that links it to growth in VSMCs. The pathways can be activated by low levels of ouabain, which do not impair cytoplasmic ionic homeostasis. When higher concentrations of the drug are used, proliferation is diminished, suggesting two functions of the drug, a proliferative effect occurring at lower concentrations and the better known pump-inhibiting effect that occurs at higher concentrations, which can interfere with the proliferative effect. The data emphasize the potential interaction between signaling pathways in complex systems as well as raise issues regarding the possible regulatory roles of endogenous digitalis-like factors.

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REFERENCES
1. Peng, M., Huang, L., Xie, Z., Huang, W. H., and Askari, A. (1996) J. Biol. Chem. 271, 10372–10378
2. Kometiani, P., Li, J., Gnudi, L., Kahn, B. B., Askari, A., and Xie, Z. (1998) J. Biol. Chem. 273, 15249–15256
3. Haendeler, J., and Berk, B. C. (2000) Regul. Pept. 95, 1–7
4. Demoliou-Mason, C. D. (1998) Biol. Signals Recept. 7, 90–97
5. Haas, M., Askari, A., and Xie, Z. (2000) J. Biol. Chem. 275, 27832–27837
6. Hamlyn, J. M., Lu, Z. R., Manunta, P., Ludens, J. H., Kimura, K., Shah, J. R., Laredo, J., Hamilton, J. P., Hamilton, M. J., and Hamilton, B. P. (1998) Clin. Exp. Hypertens. 20, 525–533
7. Allen, J. C., Navran, S. S., Seidel, C. L., Dennison, D. K., Aman, J. M., and Jemelka, S. K. (1989) Am. J. Physiol. 256, C786–C792
8. Takahashi, E., and Berk, B. C. (1998) Acta Physiol. Scand. 164, 611–621
9. Schmitz, G., Hankowitz, J., and Kovacs, E. M. (1991) Atherosclerosis 88, 109–122
10. Lompert, A. M. (1999) Clin. Exp. Pharmacol. Physiol. 26, 1–7
11. Kahn, A. M., Seidel, C. L., Allen, J. C., and Shelat, H. (1992) J. Am. Soc. Nephrol. 3, 522
12. Nakagawa, Y., Rivera, V., and Lerner, A. C. (1992) J. Biol. Chem. 267, 8785–8791
13. Golomb, E., Hill, M. R., Brown, R. G., and Keiser, H. R (1994) Am. J. Hypertens. 7, 69–74
14. Liu, J., Tian, J., Haas, M., Shapiro, J. I., Askari, A., and Xie, Z. (2000) J. Biol. Chem. 275, 27838–27844
15. Aydemir-Koksoy, A., and Allen, J. C. (2001) Am. J. Physiol 280, H1869–H1874
16. Dilerto, P. A., Gordon, G., and Herman, B. (1991) J. Biol. Chem. 266, 12612–12617
17. Saito, Y., and Berk, B. C. (2001) J. Mol. Cell. Cardiol. 33, 3–7
18. Iwasaki, H., Eguchi, S., Marumo, F., and Hirata, Y. (1988) J. Cardiovasc. Pharmacol. 31, Suppl. 1, S182–S184
19. Ishida, M., and Berk, B. C. (1997) Kase J. Med. 46, 61–68
20. Berk, B. C. (1999) J. Am. Soc. Nephrol. 10, Suppl. 11, 562–568
21. Geschwind, A., Zwick, E., Prenzel, N., Leserer, M., and Ulrich, A. (2001) Oncogene 20, 1594–1600
22. Leserer, M., Geschwind, A., and Ulrich, A. (2000) JUBMB Life 49, 405–409
23. Turner, N. A., Hall, S. G., and Balmforth, A. J. (2000) Cell. Signal. 13, 269–277
24. Danto, S. I., Borok, Z., Zhang, X. L., Lopez, M. Z., Patel, P., Crandall, E. D., and Lubman, R. L. (1998) Am. J. Physiol. 275, C82–C92
25. Ferrandis, M., Manunta, P., Rivera, E., Bianchi, G., and Ferrari, P. (1999) Am. J. Physiol. 277, H1338–H1349
26. Tripodi, G., Valtorta, F., Torielli, L., Chiergatti, E., Salardi, S., Trusolino, L., Mesegue, A., Ferrari, P., Marchisio, P. C., and Bianchi, G. (1996) J. Clin. Invest. 97, 2815–2822
27. Velarde, V., Ullian, M. E., Morinelli, T. A., Mayfield, R. K., and Jaffa, A. A. (1999) Am. J. Physiol. 277, C253–C261
28. Kranimer, H. J., Meyer-Lehnert, H., Michel, H., and Predel, H. G. (1991) Am. J. Hypertens. 481–489
29. Takahashi, H. (2000) Hypertens. Res. 23, Suppl. 1, S1–S5
30. Goto, A., Ishiguro, T., Yamada, K., Ishii, M., Yoshioka, M., Eguchi, C., Shimora, M., and Sugimoto, T. (1990) Biochem. Biophys. Res. Commun. 173, 1093–1098
31. Goto, A., Yamada, K., Yagi, N., Yoshioka, M., and Sugimoto, T. (1992) Pharmacol. Rev. 44, 377–399
32. Laredo, J., Hamilton, B. P., and Hamlyn, J. M. (1995) Biochem. Biophys. Res. Commun. 212, 487–493
