

Regulation of Na⁺ pump expression by vascular smooth muscle cells

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Aydemir-Koksoy, Aslihan, and Julius C. Allen. Regulation of Na⁺ pump expression by vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol* 280: H1869–H1874, 2001.—The Na⁺ pump and its regulation is important for maintaining membrane potential and transmembrane Na⁺ gradient in all mammalian cells and thus is essential for cell survival and function. Vascular smooth muscle cells (VSMC) have a relatively low number of pump sites on their membrane compared with other cells. We wished to determine the mechanisms for regulating the number of pump sites in these cells. We used canine saphenous vein VSMC cultured in 10% serum and passaged one time. These cells were subcultured in 5% serum media with low K⁺ (1 mM vs. control of 5 mM), and their pump expression was assessed. These VSMC upregulated their pump sites as early as 4 h after treatment (measured by [³H]ouabain binding). At this early time point, there was no detectable increase in protein expression of either α₁- or β₁-subunits of the pump shown by Western blots. When the cells were treated with the phosphoinositide 3-kinase (PI-3-K) inhibitor LY-294002 (which is known to inhibit cytoplasmic transport processes) in low-K⁺ media, the pump site upregulation was inhibited. These data suggest that the low-K⁺-induced upregulation of Na⁺ pump number can occur by translocation of preformed pumps from intracellular stores.

sodium-potassium-adenosinetriphosphatase; short-term regulation; LY-294002; low potassium

NA⁺-K⁺-ATPASE IS THE ENZYME in all mammalian cell membranes that establishes the Na⁺ gradient and the transmembrane potential. The enzyme/pump is composed of two subunits, α and β, which must exist as a dimer to establish both ion transport and enzymatic activity. The α-subunit contains the ionic and ATP binding sites and is thus designated as the catalytic subunit. The β-subunit is presumed to be important for proper structural conformation of the α-subunit. It is well known that both of the subunits have at least 3 different isoforms (6, 15). Regulators of the pump can effect transcription and/or translation of these subunits.

It has been shown that Na⁺ pump function is regulated by both short-term and long-term processes (12, 13). Short-term regulation occurs within minutes to

hours. In this process, a faster transport of ions per pump for a given time is activated through increased turnover rate of the existing pumps via protein kinase C (PKC) or protein kinase A (PKA) phosphorylation (4, 7, 19). The long-term regulation requires new mRNA and/or protein synthesis of the pump subunits and generally occurs over days (19). Studies of such pump upregulation often use agents (e.g., ouabain) or conditions (low K⁺ treatment) that inhibit pump function and challenge the cells to upregulate functional pump subunits to eliminate the increased intracellular Na⁺ (17, 20, 21). In other studies, hormones such as insulin have been shown to induce pump movement from cytoplasmic pools to the cell membrane by mechanisms that are yet to be delineated (12, 14). Although pump regulation through increased turnover and/or transcription of the α-subunit gene has been shown in vascular smooth muscle cells (VSMC; see Refs. 16 and 21), there are few studies assessing other mechanisms of regulation (24). Here, we investigated the time course of pump upregulation during the first 20 h of treatment with low-K⁺ media. We hypothesized that these cells may have a fast intermediary translocational mechanism to upregulate pump sites.

EXPERIMENTAL PROCEDURES

Materials. All chemicals, including aprotinin and ouabain, were purchased from Sigma (St. Louis, MO). [³H]ouabain was from NEN Life Science Products (Boston, MA). All media were obtained from GIBCO-BRL. LY-294002 was purchased from Calbiochem (La Jolla, CA).

VSMC culture. VSMC were isolated from the saphenous veins of mongrel dogs by a two-step enzymatic digestion as described previously (23). The collected cells were cultured in 10% serum-Dulbecco's modified Eagle's medium (DMEM) containing 5 mg/ml penicillin, 5 mg/ml streptomycin and 10 mg/ml neomycin, and 2.5 mg/ml gentamicin and 0.1 mg/ml meropenem. On reaching confluence, the cells were passaged and grown to 75% confluence in 5% serum-DMEM. At this point the medium was switched to low-K⁺ DMEM with 5% serum (final K⁺ concentration = 1.12 mM) in the treatment group. The control media contained 5 mM K⁺.

Western blots. Western blots were used to determine the expression of the α-subunit during 20 h of pump inhibition

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and were performed according to the method developed by Towbin et al. (26). After treatment in low K⁺, cells were collected by a rubber policeman on ice [by using 100 μl/100 mm lysis buffer dish (62.5 mM Tris·HCl, 2% SDS, 10 μg/ml aprotinin, 1.5 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin)]. Samples were boiled for 5 min and centrifuged for 5 min at 12,000 *g* before use. Protein (10 μg) from these samples was run on 10% SDS-polyacrylamide gels and blotted on nylon membranes. Membranes were incubated with the α₁-antibody (6F, monoclonal, courtesy of Dr. D. M. Fambrough and purchased from the Developmental Hybridoma Studies, University of Iowa) or with β₁-antibody (Upstate Biotechnology; Lake Placid, NY) at 1:1,000 dilution in blocking solution (5% dry milk in Tris buffer). The presence of the proteins was detected by the enhanced chemiluminescence plus system (Amersham Pharmacia Biotechnology; Piscataway, NJ). The density of the protein bands was analyzed by using Image Tool (University of Texas; San Antonio, TX) software.

[³H]ouabain binding. To investigate the change in pump sites, binding studies were performed with radioactive ouabain, as described earlier (3). Cells were plated in 35-mm dishes and used at 75% confluence. The cells in the treatment group were incubated with media containing 1 mM K⁺ for 2, 4, 8, 20, and 48 h. At the end of each time point, the plates were washed and incubated with ouabain-binding buffer (OBB), pH 7.4, containing (in mM) 120 NaCl, 0.05 CaCl₂, 1 MgCl₂, 5 glucose, and 2 HEPES. After 15 min of incubation in [³H]ouabain, plates were washed with OBB, and cells were collected with trypsinization to determine the cell counts in each plate. Measurement of binding in the presence of 0.25 μM [³H]ouabain (specific activity: 16.5 Ci/mmol) is used to calculate the total binding. For measuring nonspecific binding, alternate dishes contained 1 mM unlabeled ouabain. The subtraction of the latter from the former gave the specific binding. The data were expressed as picomoles [³H]ouabain bound/10⁵ cells.

RT-PCR. To determine the changes in message for the pump α-subunit, we used a Perkin-Elmer RT-PCR kit according to the method described previously (22). The primers for detecting α-subunits (5'-GTTGGACGAGACAAGTATGA-3' and 3'-CCTTGCTAAACTCGGCTCC-5') were generated earlier by our laboratory (2) and were synthesized by Genosys (Houston, TX). RNA was isolated from samples according to the method developed by Chomzynski and Sacchi (10). RNA was quantitated by spectrophotometric analysis. RNA (2.5 μg) was subjected to reverse transcription by using Maloney's murine leukemia virus RT, followed by PCR for 32 cycles by using *Taq* polymerase. The number of cycles was also determined by the cycle number that glyceraldehyde-3-phosphate dehydrogenase product approaches plateau.

Statistical analysis. We used one-way ANOVA followed by Tukey's honest significant difference test to determine the differences within groups and Student's *t*-test to analyze differences between groups.

RESULTS

[³H]ouabain binding increased significantly at and after 4 h of treatment in low-K⁺ media. We used [³H]ouabain binding as a tool to determine the number of pump sites in low-K⁺-treated cells vs. control. We observed that the pump numbers of VSMC gradually increased from 2 to 20 h, both within the low-K⁺ group and at selected time points compared with control. The increases in pump numbers were 40 and 60% over the

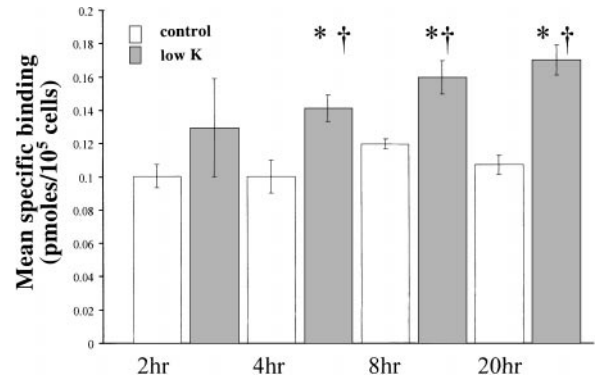


Fig. 1. [³H]ouabain binding to vascular smooth muscle cells (VSMC) treated with low-K⁺ media for 20 h. Cells were cultured to 70% confluence in 35-mm dishes in 5% serum-DMEM. Treatment group was treated with media containing 1 mM K⁺. [³H]ouabain binding increased 27, 40, 60, and 70% compared with control at 2, 4, 8, and 20 h, respectively (*n* = 7 experiments). **P* < 0.05 compared with time-matched control by Student's *t*-test. †*P* < 0.05 compared with 2-h control value by ANOVA.

control group at 4 and 8 h, respectively. The increase in bound [³H]ouabain reached 70% over control at 20 h. These observations were also valid for within-group comparison of low-K⁺ treatment when compared at different times. All of these changes were statistically significant (*P* < 0.05) compared with control (Fig. 1). In one-half of the samples, the pump numbers increased ~30–50% compared with control at 2 h, but the overall increase was not statistically significant due to the variation of response between samples at this time point (*P* = 0.2).

No change in total α₁-subunit message or protein level during the upregulation period. The canine saphenous vein cells used in this study are known to express only the α₁-isoform and its truncated form, α_{1T} (18). So far, only the α₁-isoform has been shown to participate in pump formation in these cells.

We used RT-PCR and Western blots to assess the amount of total α₁-subunit message and protein, respectively. The amount of the α₁-subunit message (Fig. 2) and protein levels (Fig. 3A) remained the same throughout the experimental period in low-K⁺-treated cells. We also observed that ouabain at concentrations that inhibited the pump to levels comparable to 1 mM K⁺ gave us the same results in α₁-protein expression (Fig. 3B). The antibody we used in our Western blots was able to detect a 10% increase in protein when the total protein loaded was >7 μg/well (Fig. 3C).¹

No change in β₁-subunit protein expression during low-K⁺ treatment. β-Subunits are necessary for assembly and transport of the Na⁺ pumps to the membrane. Canine VSMC have been shown to express β₁-subunit, which couples with α₁ to make functional pumps. For this reason, we also examined the expression of the

¹In the canine saphenous vein cultured cells used in this report, RT-PCR resulted in an extra band at the 300-bp level. Our efforts to clone this unknown band have been unsuccessful so far. This band is specific to vascular tissue, as it does not appear in samples of either heart or kidney.

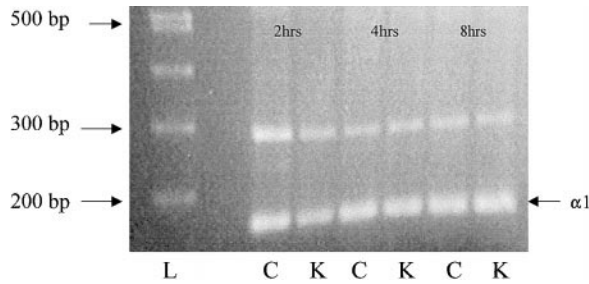


Fig. 2. RT-PCR of α_1 -subunit mRNA. Samples were collected from cells grown to 70% confluence in 60-mm dishes. The treatment group was switched to 1 mM K^+ at the time of the experiment. Samples were collected on ice, and 2.5 μ g of mRNA were subjected to reverse transcription reaction in a total of 100 μ l reaction mixture. RT-PCR was undertaken according to Perkin-Elmer's suggested protocol. The samples were subjected to 32 cycles, which is the optimum number of cycles for the given experiment determined by our laboratory previously. Each sample was compared with its own control. There was no increase in α_1 -subunit message during 20 h of treatment with low K^+ compared with controls ($n = 2$). L, ladder; C, control; K, low- K^+ -treated group.

β_1 -subunit during low- K^+ treatment. When we stripped and reprobbed the same Western blot membranes used for the detection of the α_1 -subunit with the β_1 -antibody, we saw that the expression of the β_1 -subunit also did not change (Fig. 4).

Inhibitor of phosphoinositide 3-kinase, LY-294002, inhibited upregulation of pump sites. The phosphoinositide 3-kinase (PI-3-K) pathway has been shown to play an active role in membrane transport to/from intracellular compartments and in the *trans*-Golgi network (25, 27). LY-294002 is a specific, cell-permeable, and potent inhibitor of the PI-3-K pathway. When we incubated VSMC in low- K^+ media that contained 20 μ M LY-294002 throughout the experimental period, the increase in the pump numbers was entirely inhibited compared with controls (Fig. 5). At the concentrations used in this study, LY-294002 has no other reported effects. LY-294002 did not affect the total amount of α_1 -protein in either control or K^+ -treated cells (Fig. 6).

DISCUSSION

In the present study, we assessed the pump regulation when canine VSMC are grown in a pump inhibitory medium for 20 h. Our data showed an upregulation of Na^+ pump sites without any increase in message or protein of pump subunits and suggested a cytoplasmic translocation of preformed pumps.

Previous studies in a variety of cell types under the same conditions have shown that this treatment increases intracellular Na^+ concentrations as a result of pump inhibition. The increase in intracellular Na^+ results in activation of gene transcription of the α -subunit of the pump, resulting in an increased number of pump sites (i.e., long-term regulation). There are also other ways of increasing pump function, e.g., by increasing turnover rate of existing pumps (i.e., short-term regulation) or by increasing pump site expression by translocation of preformed pumps from intracellular stores.

Short-term regulation of the pump has been shown to occur by PKA or PKC phosphorylation and subsequent activation of the α_1 -subunit. This phosphorylation event alters the turnover rate of the pump by changing its affinity for both K^+ and Na^+ (4, 5). Previously, other workers showed the translocation of

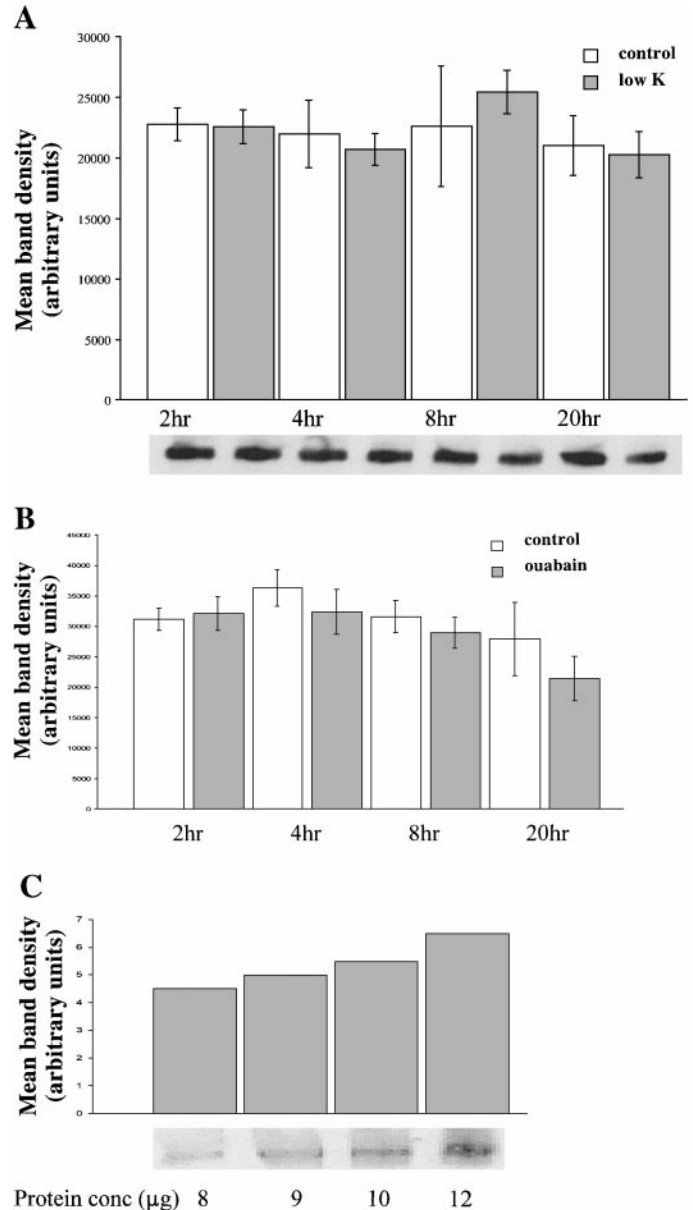


Fig. 3. A: Western blots used to determine the amount of α_1 -subunit protein expression. The amount of α_1 -protein in samples from control and low- K^+ treatment groups were determined at 2, 4, 8, and 20 h. Samples were collected on ice by using lysis buffer with 2% SDS. An aliquot containing 10 μ g of protein from each sample was subjected to 10% SDS-PAGE. There was no change in α_1 -protein expression compared with control during 20 h of low- K^+ treatment of VSMC ($n = 3$; protein expression of α_{1T} , the truncated form of the α -subunit, also remained the same during these experiments, data not shown). B: Expression of α_1 -protein during 20 h of 10^{-6} M ouabain treatment. There was no change in α_1 -protein expression with ouabain treatment for 20 h compared with controls ($n = 3$). C: 6F antibody can recognize as low as a 10% increase in α -subunit protein. Indicated concentrations of total protein were loaded to each well.

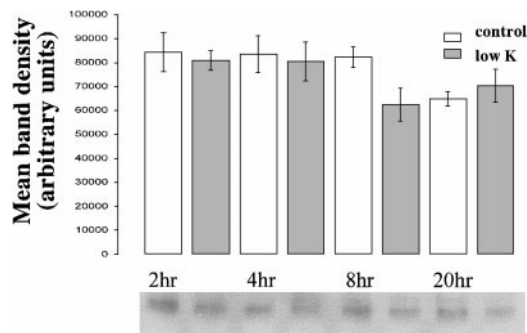


Fig. 4. Western blots to determine the amount of β_1 -subunit protein expression. The amount of β_1 -protein in samples from control and low- K^+ treatment groups were determined at 2, 4, 8, and 20 h by using the same membranes in Fig. 3A, after stripping. β_1 -Subunit expression did not change during the 20-h experimental period.

pumps from an intracellular source to the cell membrane and the reverse. For example, alveolar epithelial cells respond to isoproterenol treatment very rapidly with increased pump function and [³H]ouabain binding (5). In addition, dopamine has been shown to induce internalization of pumps in kidney epithelial cells to an endosomal compartment (9). Other studies reported a rapid increase in pump site number without an increase in protein synthesis of the pump subunits in kidney cells and in skeletal muscle after aldosterone treatment or insulin treatment, respectively (12). Preliminary studies from our laboratory suggest the existence of a drug-induced fast translocational mechanism in VSMC (somewhat similar to those of alveolar epithelial cells) shown by using adrenergic agonists (J. C. Allen and A. Bertorello, unpublished observations). Other studies by Songu-Mize et al. (24) also show a stretch-induced translocation of pumps in VSMC.

Na⁺ pump site density in VSMC has been shown to be less than that of other muscle tissues, despite the fact that the functional requirement for the pump regulation is quite similar (1). Thus it was of considerable interest to determine the nature of the pump site upregulation in this tissue. Our data have suggested

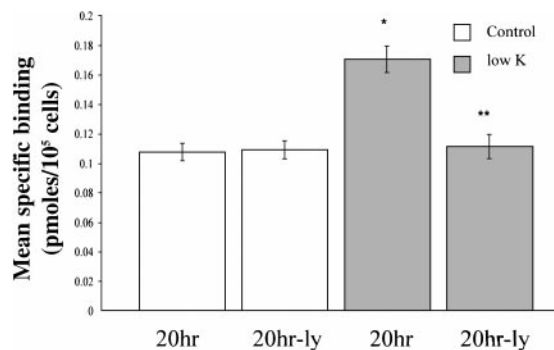


Fig. 5. LY-294002 inhibited the upregulation observed in [³H]ouabain during 20 h of low- K^+ treatment. LY-294002 (20 μ M) was given 15 min before and during the experiment. The amount of inhibition was 100% and was significantly different from the low- K^+ -treated but not LY-294002-treated group ($n = 8$). Ly, LY-294002-treated group. * $P < 0.05$ and ** $P < 0.05$, Student's t -test.

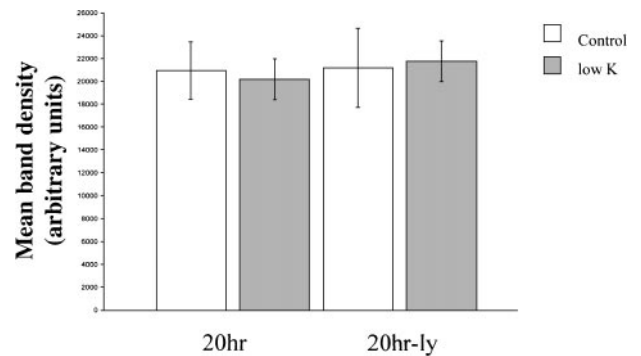


Fig. 6. LY-294002 treatment had no effect on α_1 -protein expression of cells treated with low K^+ or regular media for 20 h. LY-294002 (20 μ M) was given during 20 h of treatment with either low- K^+ or regular media (containing 5% serum; $n = 4$).

that, in VSMC, there may be mechanisms similar to those induced by stretching as well as by insulin and aldosterone in other tissues (8, 12, 21). When we treated canine VSMC in enough K^+ to inhibit the pump but still allow the cells to survive, we observed an upregulation of Na⁺ pump sites. The increase in pump site numbers measured by [³H]ouabain binding was apparent by 2 h and continued through 4 and 8 h. Other researchers using epithelial cells (8, 11) and VSMC (16, 20) showed that the earliest time that an increase in α -subunit mRNA expression was observed was after 3 h and that it continued to increase beyond 24 h. In these cases, the earliest significant increase in protein expression of the α -subunits occurred after 12 h. Some of these studies used stretch or serum to activate the regulation processes, both of which are known to increase intracellular Na⁺ and thus are similar to our study in their mechanism of triggering the cell for pump regulation. In our study, there was a very rapid increase in the number of [³H]ouabain binding sites. Because the information in the literature suggests that α -subunit mRNA accumulation in response to the mentioned stimuli reaches significant levels only after 3 h and the increase in protein expression takes place even later, we reasoned that the upregulation of pump sites that we observed may not be due to de novo pump synthesis. Although an increase in β -subunit expression alone in response to an increase in intracellular Na⁺ has not been reported in vascular smooth muscle cells before, we measured levels of total cellular α_1 - and β_1 -subunit protein and α_1 -subunit message. The Western analysis of whole cell α_1 - and β_1 -protein content at 2, 4, 8, and 20 h showed no measurable increase in the amount of either protein. Indeed, both the α_1 - and β_1 -protein content of whole cells remained constant and equal to that of controls throughout the entire experimental period, as did the protein content of the truncated subunit, α_{1T} (data not shown). The α_1 -mRNA content of these cells, determined by RT-PCR at 2, 4, 8, and 20 h, was also the same as controls. We were also able to demonstrate that ouabain at concentrations that inhibit the pump to a similar degree as 1 mM K^+ gave us the same results with respect to α_1 -protein expression. Additionally, there was no

detectable expression of α_2 - and α_3 -subunits at any level in canine VSMC (unpublished observations).

The most reasonable hypothesis to explain these observations thus far is one that considers the translocation of preformed pump sites to the cell membrane as a component of short-term pump site upregulation. The short-term regulation referred to in the literature is generally an increase in pump function that occurs within hours or less, generally because of an increased turnover rate of the pump, by the phosphorylation events referred to earlier. To test the possibility that the early pump site upregulation occurs by a translocation process, we used the well-known PI-3-K inhibitor LY-294002, an acknowledged regulator of intracellular trafficking and secretion (25, 27). In addition, a recent study by Yudowski et al. (28) showed that PI-3-K plays an important role in dopamine-induced internalization of the α -subunits in opossum kidney cells. This study showed that the proline-rich region of the Na⁺ pump α -subunit interacts with PI-3-K and that this interaction is important for its regulation (28). LY-294002 is a very potent and specific inhibitor of PI-3-K (IC₅₀ of 2 μ M). LY-294002 has no reported effect on other kinases at concentrations up to 50 μ M (27). When we used 20 μ M LY-294002 concomitant with low-K⁺ treatment, the increase in pump sites measured by [³H]ouabain binding was totally inhibited. A possible alternative explanation for these data can be that LY-294002 might interfere with ouabain binding itself. First, there were no differences between ouabain binding of cells treated with LY-294002 alone vs. control, and, in addition, there is no evidence in the literature that this compound has such an effect. Second, all of the compound was always carefully washed away before we proceeded with the ouabain-binding procedure. The analysis of binding within groups of low-K⁺-treated cells showed that the pump sites increased over the baseline during the experiment and that LY-294002 inhibited this increase and maintained ouabain binding at those baseline levels. This and the previous data together further suggest that the effect of LY-294002 cannot be due to an increase in internalization of pumps. At 20 μ M concentrations, LY-294002 had no effect on the total α_1 -protein content of VSMC. Because the mRNA expression of the α -subunit did not change with low K⁺, the effect of LY-294002 cannot be explained by gene regulation.

The data presented in this paper suggest that there appears to be an intermediate control mechanism for pump site upregulation that complements both the short- and the long-term regulation processes. Here we propose this intermediate translocational pathway to be activated when pump upregulation by increased turnover rate of the pumps would be exceeded. It is easy to imagine that this could occur quite readily in a tissue with a limited number of pump sites. Thus, in VSMC, an increase in pump activity by α_1 -subunit protein phosphorylation defines short-term regulation, cytoplasmic translocation (intermediate term regulation), and the gene transcription that takes place when

the preformed pump pool is depleted (long-term regulation).

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