J. Membrane Biol. 162, 233–245 (1998)

The Journal of
Membrane
Biology

Effects of Hyperosmolality on Na, K-ATPase Gene Expression in Vascular Smooth Muscle Cells

S. Muto, A. Ohtaka, J. Nemoto, K. Kawakami, Y. Asano
Departments of Nephrology and Biology, Jichi Medical School, Minamikawachi, Tochigi 329-04, Japan

Received: 11 July 1997/Revised: 5 January 1998

Abstract. Cultured vascular smooth muscle cells (VSMC) from rat thoracic aortas were exposed to hyperosmotic media to determine the effects on Na, K-ATPase α1- and β1-mRNA expression. Hyperosmotic media (500 mOsm/kgH_2O) supplemented with glucose or mannitol increased α1-mRNA levels threefold at 24 hr and β1-mRNA levels sevenfold at 12 hr. In sharp contrast, hyperosmotic urea medium had no effect at any time. Both the protein synthesis inhibitor cycloheximide and the RNA transcription inhibitor actinomycin D reduced α1- and β1-mRNA upregulation induced by hyperosmotic glucose or mannitol media. Protein kinase C (PKC) inhibitors (staurosporine A or calphostin C) or tyrosine kinase (TK) inhibitors (genistein or herbimycin A) had no effect on the α1-mRNA upregulation induced by hyperosmotic glucose or mannitol media. Hyperosmotic glucose or mannitol media (500 mOsm/kgH_2O) significantly increased α1- and β1-subunit protein levels and Na, K-ATPase activity, whereas hyperosmotic urea medium had no effect. Transfection experiments with the 5′-flanking sequences of the α1- or β1-subunit genes linked to the luciferase reporter gene revealed that hyperosmolar glucose medium increased luciferase activity 2.9- and 3.7-fold, respectively. Similarly, hyperosmotic mannitol medium increased such activity 2.7- and 3.4-fold, respectively. These results demonstrate that: (i) hyperosmolality induced by the poorly permeating solutes (glucose and mannitol) stimulates α1- and β1-mRNA accumulation, α1- and β1-subunit protein accumulation, and Na, K-ATPase activity, whereas the rapidly permeating solute (urea) has no effect; (ii) the upregulation of α1- and β1-mRNA in response to hyperosmotic glucose or mannitol media requires, at least in part, *de novo* synthesis of intermediate regulatory proteins; (iii) the hyperosmolality-induced α1-mRNA upregulation occurs through PKC- and TK-independent mechanisms, whereas the hyperosmolality-induced β1-mRNA upregulation occurs through activation of PKC and TK; and (iv) hyperosmolality induced by glucose or mannitol increases promoter activities of the α1- and β1-subunit genes.

Key words: glucose — mannitol — urea — protein kinase C — tyrosine kinase — hypertonicity-response elements

Introduction

Na, K-ATPase (the Na pump) is an oligometric membrane protein responsible for the primary active transport of Na and K in most animal cells. By maintaining or restoring the normal distribution of these ions across the plasma membrane, Na, K-ATPase plays a central role in the regulation of membrane potential, cell ion content, and cell volume in various kinds of cells, including vascular smooth muscle cells (VSMC) [9]. Structurally, this enzyme is composed of two subunits: a large catalytic α-subunit and a smaller glycosylated β-subunit. The α-subunit contains an intracellular ATP binding site, a phosphorylation site, and an extracellular binding site for cardiac glycosides, i.e., ouabain, whereas the β-subunit is thought to play a role in subunit assembly and in functional maturation [18]. The two subunits are encoded by multigene families, and appear to be differentially expressed among tissues and during development [26].

In rats, cDNAs encoding three α-subunit isoforms [α1, α2, α3] have been cloned and sequenced (reviewed in Refs. 18, 32). The α1-subunit is expressed ubiquitously among tissues and is thought to play a ''housekeeping'' role in maintaining Na and K gradients, whereas the α2- and α3-subunits differ in their tissue

Correspondence to: S. Muto

expression and regulation, as well as in affinity for Na and K (reviewed in Refs. 18, 32). Three β-subunit isoforms (β1, β2, β3) have also been cloned and sequenced in rats with β1 being found in all tissues, while β2 and β3 are tissue-specific [11, 20, 21]. In rat VSMC, the predominant isoforms of Na, K-ATPase are α1 and β1 (37).

When exposed to high extracellular osmolality, most cells increase their intracellular osmolyte content. This is to prevent cell dehydration. The increase in intracellular osmolality is accomplished by an increase in either NaCl influx or organic osmolyte content. Major portions of NaCl influx are mediated by Na-K-2Cl cotransport or by parallel Na/H antiport and Cl/HCO_3 antiport [4, 15]. When Na permeability is enhanced by these transport systems, Na, K-ATPase may be stimulated to maintain the intracellular Na and K concentrations [15]. Recently, upregulation of Na, K-ATPase α1- and β1-mRNA expression in response to hyperosmotic media has been reported in Madin-Darby canine kidney (MDCK) cells [2] and in cultured inner medullary collecting duct (IMCD) cells from rat kidneys [24]. Renal-medulla kidney cells are normally the only cells in mammals that are exposed to elevated and fluctuating ambient osmolality as a consequence of urinary concentrating mechanisms. However, under pathological conditions, such as diabetes mellitus or uremia, elevated hyperosmotic conditions exist in the blood (caused by elevated glucose or urea levels, respectively). It is not yet known whether these hyperosmotic agents influence Na, K-ATPase gene expression in vascular tissues. Determining the effects of hyperosmolality on Na, K-ATPase gene expression in vascular tissues might be of clinical importance, since modulation of Na, K-ATPase gene expression may contribute to cell volume regulation of vascular cells in patients with diabetes mellitus or uremia. Therefore, we prepared VSMC from rat thoracic aortas to address the following issues: (i) whether hyperosmolality induced by glucose, mannitol, or urea stimulates Na, K-ATPase α1- and β1-mRNA expression, α1- and β1-subunit protein accumulation, and Na, K-ATPase activity in VSMC; (ii) what the mechanisms are for the upregulation of α1- and β1-mRNA induced by hyperosmolality; and (iii) whether the 5′-flanking regions of the α1- and β1-subunit genes are hyperosmolality responsive.

Materials and Methods

Culture of Rat VSMC

Primary cultures of rat VSMC were isolated from the media of thoracic aortas of male Sprague-Dawley rats (200–250 g) by treatment with collagenase, as previously described [22]. Cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 26.1 mM $NaHCO_3$, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 incubator at 37°C. After reaching confluence, VSMC were passaged through trypsin/EDTA and seeded at 1:3 ratio. All experiments were performed with VSMC which were grown to confluence after 4–10 passages.

Northern Blot Analysis

Total RNA was extracted from 100-mm dishes (Becton Dickinson, NJ) as described previously in our laboratory [22, 24]. The RNA was fractionated on 1% denaturing formaldehyde/agarose gels and transferred onto nylon membrane filters (Hybond N, Amersham, Bucks, UK) by capillary blotting overnight. Filters were then hybridized with cDNAs encoding the rat Na, K-ATPase α1 or β1. The α1-cDNA probe consisted of a 2.2 kb *Nco* I restriction fragment [15]. The β1-cDNA probe consisted of a 0.9 kb *Pst* I-*Spe* I restriction fragment [39]. Probes were labeled with [α-^{32}P]dCTP using an Amersham Multiprime DNA labeling kit (Amersham). The filters were prehybridized, hybridized, and washed as described previously [22, 24]. The filters were then exposed to autoradiographic film with an intensifying screen at −70°C. The mRNA was quantitated by densitometric scanning (Image Analyzer TIF-64, Immunomedica, Tokyo, Japan). The filters were also hybridized with [γ-^{32}P]ATP-labeled rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) oligonucleotide probe. The α1- and β1-mRNA areas were normalized to the GAPDH area for each lane to control for variability in RNA loading.

Measurement of [^{3}H]-Leucine Incorporation

[^{3}H]-leucine incorporation into proteins was determined in VSMC to assess the efficiency of cycloheximide treatment. For this purpose, VSMC were plated in 24-well plates (Corning, NY) in MEM containing 10% serum. Confluent cells were then preincubated at 37°C for 48 hr in leucine- and serum-free MEM. The protein synthesis inhibitor cycloheximide at a concentration of 20 μg/ml was then added to the MEM. Thirty min later [^{3}H]-leucine, 2 μCi, was added to the medium. Control cells were prepared in [^{3}H]-leucine in leucine-free MEM without cycloheximide. After 12-hr incubation, cells were washed three times with ice-cold phosphate buffered saline (PBS), fixed by washing twice with ice-cold 10% perchloric acid, and extracted with 0.5 N NaOH-0.1% Triton X-100. Aliquots were counted in a liquid scintilation counter (LSC-3500, Aloka, Tokyo, Japan). Cellular protein content was determined by applying the Bio-Rad microassay method (Bio-Rad Laboratories, Richmond, CA), using bovine serum albumin as the standard.

Western Blot Analysis

Confluent VSMC were incubated in serum-free MEM for 48 hr, and were then treated with MEM (300 mOsm/kgH_2O) or hyperosmotic MEM (500 mOsm/kgH_2O) supplemented with glucose, mannitol, or urea for the next 24 hr. They were washed three times with ice-cold PBS, scraped, and centrifuged at 1,600 × *g* for 3 min at 4°C. The supernatant was removed, and cell pellets were stored at −70°C before use. Homogenization was performed by sonication with Branson sonifier (Branson Ultrasonics, Danbury, CT) at settings of 3 40% ducy cycle, 20 pulses in homogenization buffer (250 mM sucrose, 5 mM NaN_3, 2 mM EGTA, 200 μM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin A, and 20 mM Hepes pH 7.4). Homogenate extracts were centrifuged for 3 min at 1,600 × *g* at 4°C to pellet unbroken cells, and the resulting supernatant was collected for use in immunoblotting. The protein content of the cells was also determined by the Bio-Rad protein assay kit (Bio-Rad).

Homogenate protein (20 μg) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on polyacrylamide gels (9.0% for α1, and 10.5% for β1), and then electrophoretically blotted onto nitrocellulose membranes (Hybond-ECL, Amersham). Uniform blotting across the gel was verified by Coomasie brilliant blue staining of the post-blot gel. The transfer membranes were incubated for 1 hr at room temperature with Tris buffered saline-Tween 20 (TBS-Tween; 150 mM NaCl, 50 mM Tris/HCl, pH 7.5, 0.1% Tween 20) containing 5% (w/v) skim milk, followed by a 1hr-incubation with the desired antibody, i.e., rabbit anti-rat Na, K-ATPase α1- or β1-specific antibodies (Upstate Biotechnology, Lake Placid, NY). The antibodies were diluted in TBS-Tween (1:200 for α1, or 1:1,000 for β1). After incubation, the membranes were washed three times for 15 min each in TBS-Tween at room temperature, and were then incubated for 1 hr with horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G antibody (1:5,000 dilution) (Amersham). The membranes were again washed three times for 15 min each in TBS-Tween at room temperature, and were then made visible by fluorography with enhanced chemiluminescence detection kit (Amersham). The results were quantitated by densitometric scanning (Immunomedica), and expressed as relative increase compared with control. The reliability of the kit for quantitation of protein abundance was determined. We loaded 10, 20, and 40 μg cell homogenate and resolved by SDS-PAGE. The densitometer scanned immunoblotting results of the above samples were plotted and revealed a satisfactory linearity.

Measurement of Na, K-ATPase Activity

VSMC were cultured and grown in 60 mm culture dishes (Becton Dickinson). Confluent VSMC were incubated in serum-free MEM for 48 hr. They were then exposed to MEM (300 mOsm/kgH_2O) or hyperosmotic MEM (500 mOsm/kgH_2O) supplemented with glucose, mannitol, or urea. After 24- or 48-hr incubation, they were rinsed three times with ice-cold PBS, scraped, and centrifuged at 1,600 × *g* for 3 min at 4°C. After the supernatant was aspirated, the cells were hypotonically lysed in 1 mM Tris-HCl, pH 7.4, at 4°C, followed by mechanical disruption by sonication with Branson sonifier (Branson Ultrasonics) at settings of 3 40% duty cycle, 20 pulses. Homogenate extracts were then centrifuged for 3 min at 1,600 × *g* at 4°C to pellet unbroken cells, and the resulting supernatant was collected for use in Na, K-ATPase activity.

The method used for the determination of Na, K-ATPase activity is similar to that previously described by Garg et al., [10] with some modifications. The ATPase assay couples the hydrolysis of ATP to ADP to the oxidation of NADH. Cell homogenates prepared above were divided into two parts that were incubated with or without 4 mM ouabain at 37°C for 15 min in the following incubation medium: 100 mM NaCl, 67 mM NH_4Cl, 50 mM imidazole, 3.7 mM $MgCl_2$, 1.1 mM Na_2ATP, 0.8 mM phosphoenolpyruvate, 2.5 mM sodium azide and 0.01 mM NADH, in addition to 3.2 U/ml pyruvate kinase and 4.1 U/ml lactate dehydrogenase, pH 7.4. The fluorescence was measured using a fluorometer (RF-5000, Shimadzu Corporation, Kyoto, Japan) with excitation at 340 nm and emission at 460 nm. Na, K-ATPase activity was equivalent to the activity measured without ouabain minus the activity measured in the presence of ouabain, and expressed as μmol/mg protein/hr. The protein content of the cells was determined by the Bio-Rad protein assay kit (Bio-Rad).

Plasmid Constructs

The pA1LF-1, which harbors the *Sal* I-*Nco* I fragment of the rat Na, K-ATPase α1-subunit gene from the 5′-flanking region (−1,537) to just before the translation initiation site in the first exon (+261) fused to the firefly luciferase coding sequence in the plasmid pSV0A/LΔ5′, was used for the reporter gene assay as previously described [31]. The pSV0A/LΔ5′ construct contained the entire coding sequence of the luciferase gene minus its promoter [8]. The DNA fragment from −764 to +129 of the rat Na, K-ATPase β1-subunit gene was isolated from the rat genomic DNA by PCR with primers of CCCTCGAGACGGGAAGAGCCACAGAC and CCAAGCTTACCACTCTGCCTGCTATGACCT. The PCR product was digested with *Xho* I and *Hind* III, and subcloned into pSV0A/LΔ5′ (pB1LF-1). Both plasmids were purified using Qiagen columns (Qiagen, Chatworth, CA) according to the manufacturer's instructions.

Transfection Experiments

VSMC were cultured in 60-mm dishes (Becton Dickinson) and transfected with 5 μg of pSV0A/LΔ5′, pA1LF-1, or pB1LF-1 per dish by the calcium phosphate coprecipitation method [5]. After a 24-hr incubation, VSMC were washed with PBS, and were then exposed to MEM (300 mOsm/kgH_2O) or hyperosmotic MEM (500 mOsm/kgH_2O) supplemented with glucose, mannitol, or urea for the next 24 hr. The cells were then harvested with 250 μl of cell lysis buffer containing 100 mM potassium phosphate (pH 7.8), 1 mM dithiothreitol, and 0.2% Triton X-100. Cell extracts were centrifuged at 16,000 × *g* for 2 min at 4°C, and luciferase activity was measured. For this purpose, a 20-μl aliquot was mixed at room temperature with 100 μl of luciferase assay reagent (PicaGene luciferase assay system, Toyo B-Net, Tokyo, Japan) containing firefly luciferin. Emission of light, integrated over 10 sec, was measured with a luminometer (AutoLumat LB 953; EG&G Berthold, MA). The luciferase activity was normalized with cell protein content, and was expressed as -fold increase over control medium. The protein content of the cells was also determined by a protein assay kit (Bio-Rad).

Chemicals

All chemicals were obtained from Sigma Chemical (St. Louis, MO) unless otherwise noted as follows: MEM, penicillin, streptomycin, and FBS were from Flow laboratories (Irvine, Scotland); rabbit anti-rat Na, K-ATPase α1- and β1-subunit specific polyclonal antibodies were from Upstate Biotechnology; [α-^{32}P]dCTP, [γ-^{32}P]ATP, and [^{3}H]-leucine were from Amersham; rat GAPDH probe was from Oncogene Science (Uniondale, NY); and Na_2ATP, NADH, pyruvate kinase, lactate dehydrogenase, and phosphoenolpyruvate were from Boehringer Mannheim (Mannheim, Germany).

Statistical Analysis

Values are expressed as the means ± SE. Comparisons were performed by nonpaired Student's *t*-test, Mann-Whitney's U-test, or one-way analysis of variance in combination with Schefe's multiple comparison test, as needed. *P* values less than 0.05 were considered to be significant.

Results

Effects of Hyperosmolality on Na, K-ATPase α1- and β1-mRNA Accumulation

We first examined whether hyperosmolality induces Na, K-ATPase gene expression in VSMC. VSMC were

placed in serum-free MEM for 48 hr, and were then exposed to MEM (300 mOsm/kgH_2O, control) or hyperosmotic MEM (500 mOsm/kgH_2O) supplemented with glucose, mannitol, or urea for the next 48 hr. Representative Northern blot analyses of α1- and β1-mRNA accumulation in hyperosmolar glucose medium are shown in Fig. 1*A*, and quantitations of each mRNA amount are summarized in Fig. 1*B*. When VSMC were incubated in a hyperosmotic medium supplemented with glucose, α1-mRNA levels gradually increased to 2.7 times the control level as early as 6 hr, with a maximum 3.4-fold elevation at 24 hr, and then slightly decreased to 3.0 times the control level at 48 hr. On the other hand, β1-mRNA levels increased to 4.3 times the control level as early as 6 hr, with a maximum 6.9-fold increase at 12 hr, and then decreased to 5.0 times the control level at 48 hr. Control medium caused no effect at any time. These effects are not specific for glucose-supplemented medium. Similar findings were also observed in VSMC treated with a hyperosmotic mannitol medium. α1-mRNA levels gradually increased to 2.1 times the control level as early as 6 hr, with a maximum 3.3-fold elevation at 24 hr, and then slightly decreased to 3.1 times the control level at 48 hr (Fig. 2*A* and *B*). β1-mRNA levels increased to 4.5 times the control level as early as 6 hr, with a maximum 7.1-fold increase at 24 hr, and then decreased to 3.5 times the control level at 48 hr (Fig. 2*A* and *B*). Control cells showed relatively constant levels of both α1- and β1-mRNA throughout the time course. In sharp contrast, hyperosmolar urea medium did not stimulate either α1- or β1-mRNA levels at any time points, as shown in Fig. 3*A* and *B*. Therefore, α1- and β1-mRNA levels are upregulated in a time-dependent fashion by hyperosmotic media treated with solutes other than urea, indicating that increased osmolality per se is not sufficient for induction of Na, K-ATPase gene in response to hyperosmotic stress.

To examine the osmolality-dependent effects on of α1- and β1-mRNA accumulation, VSMC were incubated for 12 hr in hyperosmotic media with osmolalities ranging from 300 up to 550 mOsm/kgH_2O. As shown in Fig. 4, when VSMC were exposed to hyperosmotic media treated with glucose or mannitol, maximal effects on both α1- and β1-mRNA occurred at 500 mOsm/kgH_2O. By contrast, treatment of VSMC with urea produced no effect at any level of osmolality (Fig. 4). Therefore, addition of hyperosmolar glucose or mannitol media increased α1- and β1-mRNA levels in an osmolality-dependent fashion.

We next examined whether the hyperosmolality-induced upregulation of α1- and β1-mRNA occurs through newly synthesized intermediate regulatory proteins. The protein synthesis inhibitor, cycloheximide, was added to the MEM at a concentration adequate to completely block protein synthesis; in the present study,

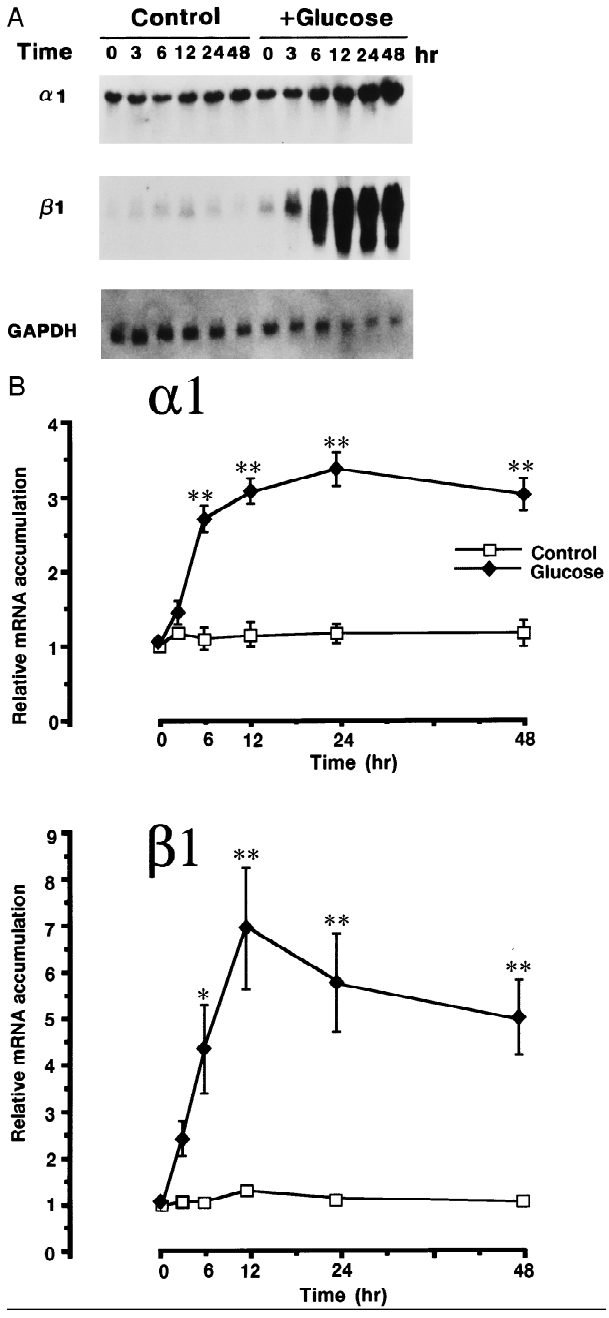


Fig. 1. Time course of Na, K-ATPase α1- and β1-mRNA accumulation in VSMC treated with MEM (Control, 300 mOsm/kgH_2O) or hyperosmotic MEM treated with glucose (+Glucose, 500 mOsm/kgH_2O). (*A*) Northern blot analyses. (*B*) Quantitations of the Northern blots. Abundance of mRNA is expressed as units of densitometry relative to *time 0;* each point is means ± SE of 3 separate experiments. **P* < 0.05, ***P* < 0.001 compared to control.

20 μg/ml cycloheximide inhibited protein synthesis by 95% as determined by [^{3}H]leucine incorporation (*data not shown*). For this purpose, VSMC were preincubated for 30 min with cycloheximide, and were further exposed to MEM (300 mOsm/kgH_2O, control) or hyperosmotic

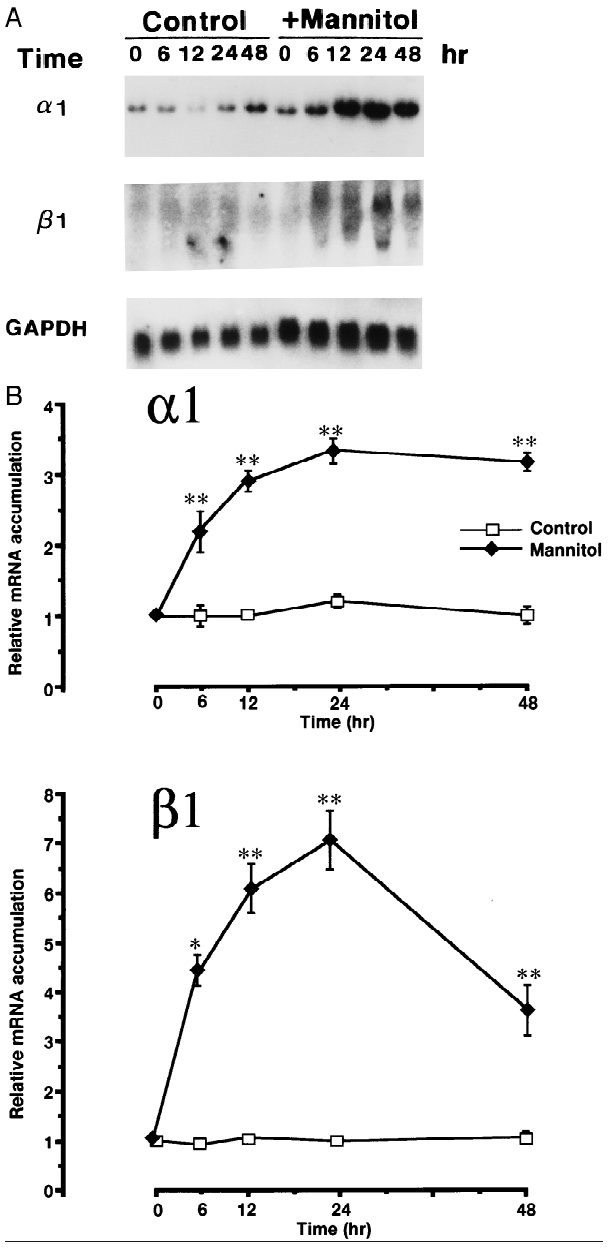


Fig. 2. Time course of Na, K-ATPase α1- and β1-mRNA accumulation in VSMC treated with MEM (Control, 300 mOsm/kgH_2O) or hyperosmolar MEM treated with mannitol (+Mannitol, 500 mOsm/kgH_2O). (*A*) Northern blot analyses. (*B*) Quantitations of the Northern blots. Abundance of mRNA is expressed as units of densitometry relative to *time 0;* each point is means ± SE of 3 separate experiments. **P* < 0.05, ***P* < 0.001 compared to control.

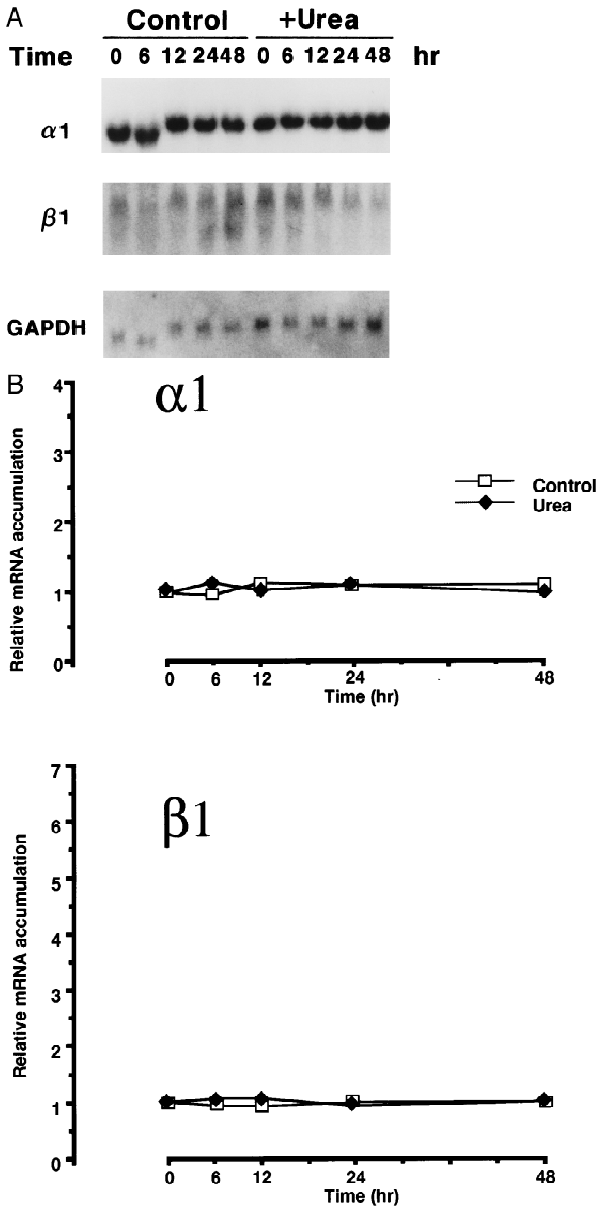


Fig. 3. Time course of Na, K-ATPase α1- and β1-mRNA accumulation in VSMC treated with MEM (Control, 300 mOsm/kgH_2O) or hyperosmotic MEM treated with urea (+Urea, 500 mOsm/kgH_2O). (*A*) Northern blot analyses. (*B*) Quantitations of the Northern blots. Abundance of mRNA is expressed as units of densitometry relative to *time 0;* each point is means ± SE of 3 separate experiments.

MEM (500 mOsm/kgH_2O) supplemented with glucose or mannitol in the continued presence of cycloheximide for 12 hr. Representative Northern blots are shown in Fig. 5*A*, and quantitations of the Northern blots are summarized in Fig. 5*B*. As previously described [23], cycloheximide alone significantly increased α1-mRNA levels, whereas it had no effect on β1-mRNA levels. Hyperosmotic glucose or mannitol media significantly enhanced α1- or β1-mRNA levels. The stimulatory effects of hyperosmotic glucose or mannitol media on α1- or β1-mRNA accumulation were inhibited by pretreatment with cycloheximide.

Since mRNA levels can rise as the result of either an increase in the rate of transcription or a decrease in the rate of degradation, the effect of the mRNA transcription inhibitor actinomycin D was examined. For this pur-

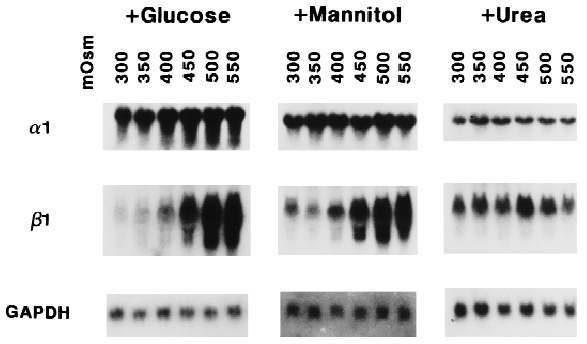


Fig. 4. Osmolality-dependent effects on Na, K-ATPase α1- and β1-mRNA accumulation in VSMC. Glucose (+Glucose), mannitol (+Mannitol), or urea (+Urea) were added to the MEM in amounts ranging from 300 up to 550 mOsm/kgH_2O for 12 hr. Each blot is a representative result of 3 separate experiments.

pose, VSMC were preincubated for 1 hr with actinomycin D (5 μg/ml) and were then further exposed to MEM (300 mOsm/kgH_2O) or hyperosmotic MEM (500 mOsm/kgH_2O) supplemented with glucose or mannitol in the continued presence of actinomycin D for 12 hr. As shown in Fig. 6, actinomycin D completely abolished the stimulatory effects on α1- or β1-mRNA levels induced by hyperosmotic glucose or mannitol media.

Hyperosmolality is known to activate protein kinase C (PKC), and finally mitogen-activated protein (MAP) kinase in yeast [3] and in MDCK cells [16, 34]. Therefore, we next examined whether hyperosmolality-induced upregulation of α1- and β1-mRNA occurs via activation of PKC. VSMC were placed in serum-free MEM for 48 hr, and were then exposed to the medium treated with one of two PKC inhibitors (staurosporine A: 0.1 μM/ml, or calphostin C: 0.5 μg/ml), plus either hyperosmotic media (500 mOsm/kgH_2O) supplemented with glucose or mannitol for 12 hr. Representative Northern blot analyses are shown in Fig. 7*A* (for glucose-supplemented medium) and *B* (for mannitol–supplemented medium). Staurosporine A or calphostin C alone caused no effect on α1- or β1-mRNA accumulation. Hyperosmotic media treated with glucose or mannitol elevated α1- and β1-mRNA levels 2.5- to 3.1-fold and 6.0- to 7.5-fold, respectively. Addition of staurosporine A or calphostin C had no effect on hyperosmolar glucose- or mannitol-induced upregulation of α1-mRNA. In sharp contrast, treatment of VSMC with staurosporine A or calphostin C significantly reduced the hyperosmolar glucose-induced upregulation of β1-mRNA to 1.7 and 3.9 times the control level, respectively. Similarly, inhibition with staurosporine A or calphostin C significantly reduced the hyperosmolar mannitol-induced upregulation of β1-mRNA to 1.8 and 4.4 times the control level, respectively.

Hyperosmolality is also known to activate MAP kinase via tyrosine phosphorylation of *HOG1,* which encodes members of the MAP kinase gene families in yeast and in mammalian cells [13]. We therefore examined whether hyperosmolality-induced upregulation of α1- or β1-mRNA occurs via activation of tyrosine kinase (TK). VSMC were placed in serum-free MEM for 48 hr, and were then exposed to the medium treated with one of two TK inhibitors (genistein: 30 μg/ml, or herbimycin A: 1 μg/ml), plus either hyperosmotic MEM (500 mOsm/kgH_2O) supplemented with glucose or mannitol for 12 hr. Representative Northern blot analyses are shown in Fig. 8*A* (for glucose supplemented medium) and *B* (for mannitol supplemented medium). Genistein or herbimycin A alone produced no effect on α1- or β1-mRNA accumulation. Hyperosmotic media treated with glucose or mannitol caused an increase in α1- and β1-mRNA levels 2.7- to 3.0-fold and 6.0- to 7.5-fold, respectively. Addition of genistein or herbimycin A had no effect on hyperosmolar glucose- or mannitol-induced α1-mRNA upregulation. In sharp contrast, treatment with genistein or herbimycin A significantly reduced the hyperosmolar glucose-induced β1-mRNA upregulation to 1.6 and 3.7 times the control level, respectively. Similarly, inhibition with genistein or herbimycin A significantly reduced the hyperosmolar mannitol-induced β1-mRNA upregulation to 3.3 and 3.8 times the control level, respectively.

We previously reported that hyperosmolality-induced upregulation of Na, K-ATPase gene expression requires extracellular Na in renal IMCD cells [24]. We next examined whether hyperosmolality-induced α1- and β1-mRNA upregulation occurs through Na entry into the cell. Since Na/H antiport [25, 30] and Na-K-2Cl cotransport [25] are influx pathways of Na into VSMC and are activated by hyperosmolality [25, 30], we used specific inhibitors of Na/H antiport (100 μM ethylisopropylamiloride) and Na-K-2Cl cotransport (10^{-4} M bumetanide) to observe the effects of hyperosmolality-induced upregulation of α1- and β1-mRNA. However, neither of the two inhibitors inhibited hyperosmolality-induced α1- and β1-mRNA upregulation (*data not shown*). We further examined the effects of removal of Na from the extracellular medium (replacement with choline) on hyperosmolality-induced upregulation of α1- and β1-mRNA. Removal of Na also had no effects on hyperosmotic glucose- or mannitol-induced upregulation of α1- and β1-mRNA (*data not shown*).

Effects of Hyperosmolality on α1- and β1-Subunit Protein Accumulation

We next examined whether hyperosmolality (500 mOsm/kgH_2O) induced by glucose, mannitol, or urea stimulates α1- and β1-subunit protein accumulation in VSMC. As shown in Fig. 9, when VSMC were treated with hyperosmolar glucose medium for 24 hr, α1- and β1-subunit protein levels increased 2.1- and 1.8-fold, re-

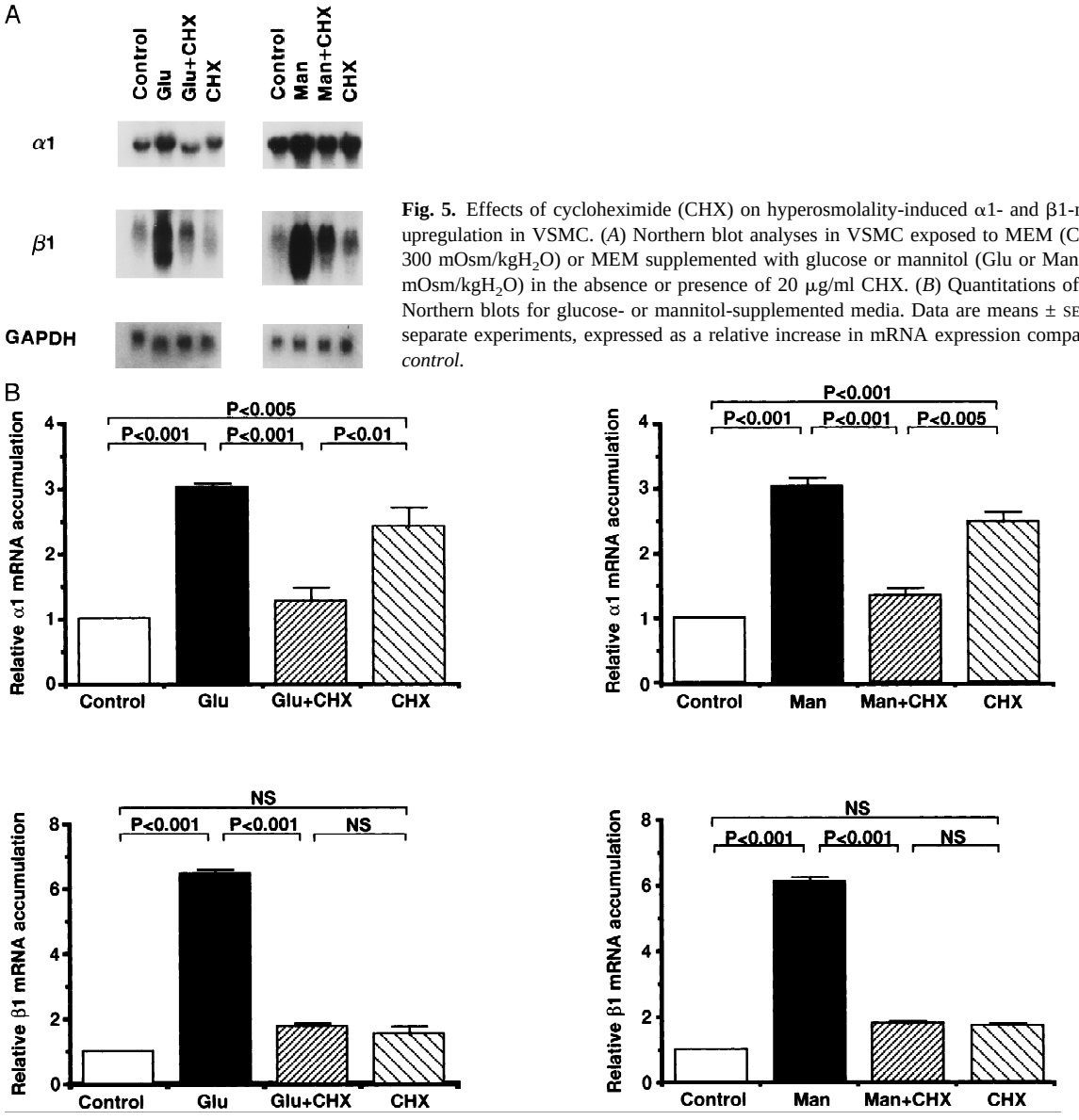


Fig. 5. Effects of cycloheximide (CHX) on hyperosmolality-induced α1- and β1-mRNA upregulation in VSMC. (*A*) Northern blot analyses in VSMC exposed to MEM (Control, 300 mOsm/kgH_2O) or MEM supplemented with glucose or mannitol (Glu or Man, 500 mOsm/kgH_2O) in the absence or presence of 20 μg/ml CHX. (*B*) Quantitations of the Northern blots for glucose- or mannitol-supplemented media. Data are means ± SE of 3 separate experiments, expressed as a relative increase in mRNA expression compared with *control.*

spectively. By contrast, treatment with hyperosmolar urea medium had no effect on α1- or β1-subunit protein accumulation.

Effects of Hyperosmolality on Na, K-ATPase Activity

We next examined whether hyperosmolality (500 mOsm/kgH_2O) induced by glucose, mannitol, or urea increases Na, K-ATPase activity (Fig. 10). When VSMC were exposed to hyperosmotic glucose medium for 24 or 48 hr, Na, K-ATPase activity increased 1.4- and 1.9-fold, respectively. Similarly, when VSMC were exposed to hyperosmotic mannitol medium for 24 or 48 hr, Na, K-ATPase activity increased 1.5- and 2.0-fold, respectively. In sharp contrast, 24- or 48-hr exposure of VSMC to hyperosmotic urea medium had no effect on Na, K-ATPase activity.

Effects of Hyperosmolality on Promoter Activities in Transfected VSMC

Finally, we examined whether the 5′-flanking regions of the α1- and β1-subunit genes is hyperosmolality responsive. For this purpose, chimeric plasmids containing the 5′-flanking sequences of the α1- or β1-subunit genes (pA1LF-1 or pB1LF-1, respectively) or plasmid alone (pSV0A/LΔ5′) were transiently transfected into VSMC and a luciferase reporter gene assay was performed (Fig. 11). pSV0A/LΔ5′ had essentially no luciferase activity

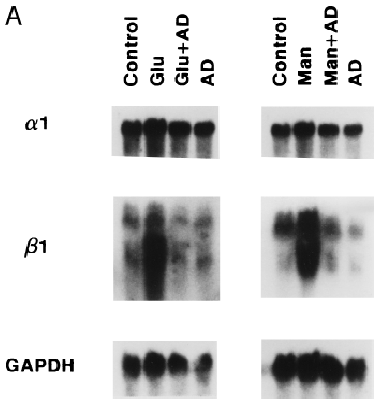
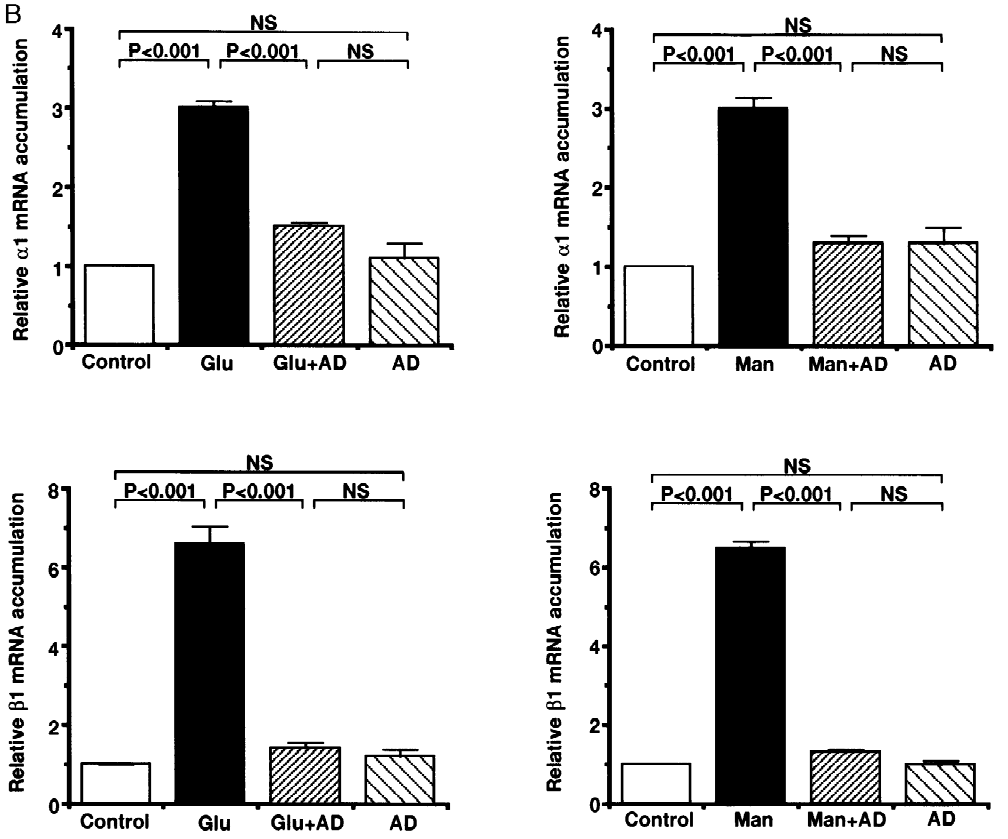


Fig. 6. Effects of actinomycin D (AD) on hyperosmolality-induced α1- and β1-mRNA upregulation in VSMC. (*A*) Northern blot analyses in VSMC exposed to MEM (Control, 300 mOsm/kgH_2O) or MEM supplemented with glucose or mannitol (Glu or Man, 500 mOsm/kgH_2O) in the absence or presence of 5 μg/ml AD. (*B*) Quantitations of the Northern blots for glucose- or mannitol-supplemented media. Data are means ± SE of 3 separate experiments, expressed as a relative increase in mRNA expression compared with *control.*



in transfected VSMC exposed to MEM or hyperosmotic MEM (500 mOsm/kgH_2O) supplemented with glucose, mannitol, or urea (*data not shown*). On the other hand, hyperosmolar glucose medium enhanced luciferase activities for the α1- and β1-subunit genes 2.9- and 3.7-fold, respectively. Similarly, hyperosmotic mannitol medium increased luciferase activities for the α1- and β1-subunit genes 2.7- and 3.4-fold, respectively. In sharp contrast, hyperosmotic urea medium had no effect on luciferase activities for the α1- or β1-subunit genes.

Discussion

The goal of this study was to investigate whether hyperosmolality affects Na, K-ATPase gene expression in VSMC. We demonstrated that in VSMC, hyperosmolality induced by glucose or mannitol stimulates α1- and β1-mRNA accumulation, α1- and β1-subunit protein accumulation, and Na, K-ATPase activity, whereas hyperosmolality induced by urea lacks these stimulatory effects. We also found that α1- and β1-mRNA upregulation induced by hyperosmolar glucose or mannitol media requires, at least in part, *de novo* synthesis of intermediate regulatory proteins, and occurs mainly due to increased mRNA production. The present study also found that the α1-mRNA upregulation induced by hyperosmolar glucose or mannitol media occurs independently of PKC and TK, whereas the β1-mRNA upregulation induced by these same media occurs through activation of PKC and TK. Furthermore, hyperosmolar

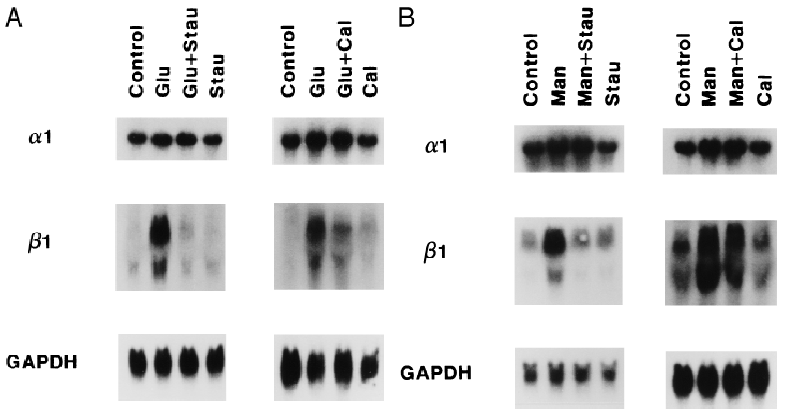


Fig. 7. Effects of PKC inhibitors, staurosporine A (Stau) or calphostin C (Cal), on hyperosmolality-induced α1- and β1-mRNA upregulation in VSMC. (*A*) Northern blot analyses in VSMC exposed to MEM (Control, 300 mOsm/kgH_2O) or MEM supplemented with glucose (Glu, 500 mOsm/kgH_2O) in the absence or presence of Stau or Cal. Results are representative of 4 separate experiments. (*B*) Northern blot analyses in VSMC exposed to MEM (Control, 300 mOsm/kgH_2O) or MEM supplemented with mannitol (Man, 500 mOsm/kgH_2O) in the absence or presence of Stau or Cal. Results are representative of 4 separate experiments.

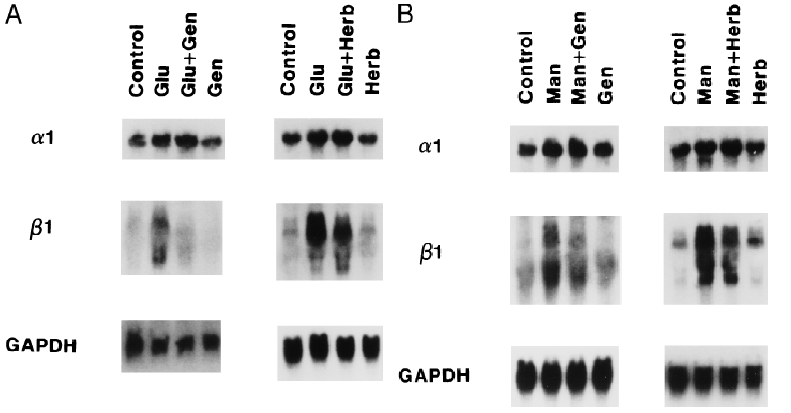


Fig. 8. Effects of TK inhibitors, genistein (Gen) or herbimycin A (Herb) on hyperosmolality-induced α1- and β1-mRNA upregulation in VSMC. (*A*) Northern blot analyses in VSMC exposed to MEM (Control, 300 mOsm/kgH_2O) or MEM supplemented with glucose (Glu, 500 mOsm/kgH_2O) in the absence or presence of Gen or Herb. Results are representative of 4 separate experiments. (*B*) Northern blot analyses in VSMC exposed to MEM (Control, 300 mOsm/kgH_2O) or MEM supplemented with mannitol (Man, 500 mOsm/kgH_2O) in the absence or presence of Gen or Herb. Results are representative of 4 separate experiments.

glucose or mannitol increases promoter activities of the α1- and β1-subunit genes.

Upregulation of Na, K-ATPase mRNA can involve either coordinate or noncoordinate changes in α- and β-mRNA with respect to time and/or magnitude; in this study hyperosmotic glucose- or mannitol-supplemented media increased α1- and β1-mRNA accumulation noncoordinately with respect to both time and magnitude. In both types of media, a maximum increase in β1-mRNA occurred at early time points, whereas the peak increase in α1-mRNA occurred at later time points. β1-mRNA levels in glucose- or mannitol-treated VSMC were much higher than α1-mRNA levels. Similar findings have been reported in dexamethasone- or aldosterone-treated VSMC [22]. This noncoordinate regulatory pattern has also been reported in other cell systems. In cultured mesangial cells, T3 induces a more rapid increase in α1-mRNA levels than in β1-mRNA levels [23]. Furthermore, changes in α- and β-mRNA abundance under hyperosmotic conditions are regulated depending on cell type. In primary cultures of human renal cortex cells exposed to hyperosmotic medium, increases in β-mRNA are greater than those in α-mRNA at early time points [38]. When MDCK cells are exposed to hypertonic media, α- and β-mRNA levels increase coordinately with respect to both time and magnitude [2]. In renal IMCD cells [24], hyperosmolality increases α1- and β1-mRNA accumulation in a noncoordinate manner with respect to magnitude, so that relative abundance of β1-mRNA is much greater than that of α1-mRNA, although α1- and β1-mRNA accumulation are raised coordinately over time. Therefore, expression of Na, K-ATPase α- and β-mRNA may be differently regulated depending on cell type and stimuli. However, the mechanisms that give rise to these variations in patterns of subunit expression are not yet known.

The present study demonstrates that in VSMC, hyperosmolar glucose or mannitol media stimulate α1- and β1-mRNA accumulation, α1- and β1-subunit protein accumulation, and Na, K-ATPase activity, whereas hyperosmolar urea medium causes no effect. Since glucose and mannitol are relatively impermeant, it is most likely that they make the medium hypertonic. In contrast, urea is freely permeant, and raises the osmolality but does not affect the medium tonicity [35]. Accordingly, medium tonicity — rather than absolute osmolality — is the important factor for α1- and β1-mRNA accumulation, α1- and β1-subunit protein accumulation, and Na, K-ATPase activity. Similar findings have been reported in renal IMCD cells [24]: hyperosmolar NaCl, mannitol, or raffinose media raised α1- and β1-mRNA accumulation, although hyperosmolar urea medium had no effect.

We demonstrated that in VSMC, cycloheximide alone significantly increases α1-mRNA levels but not

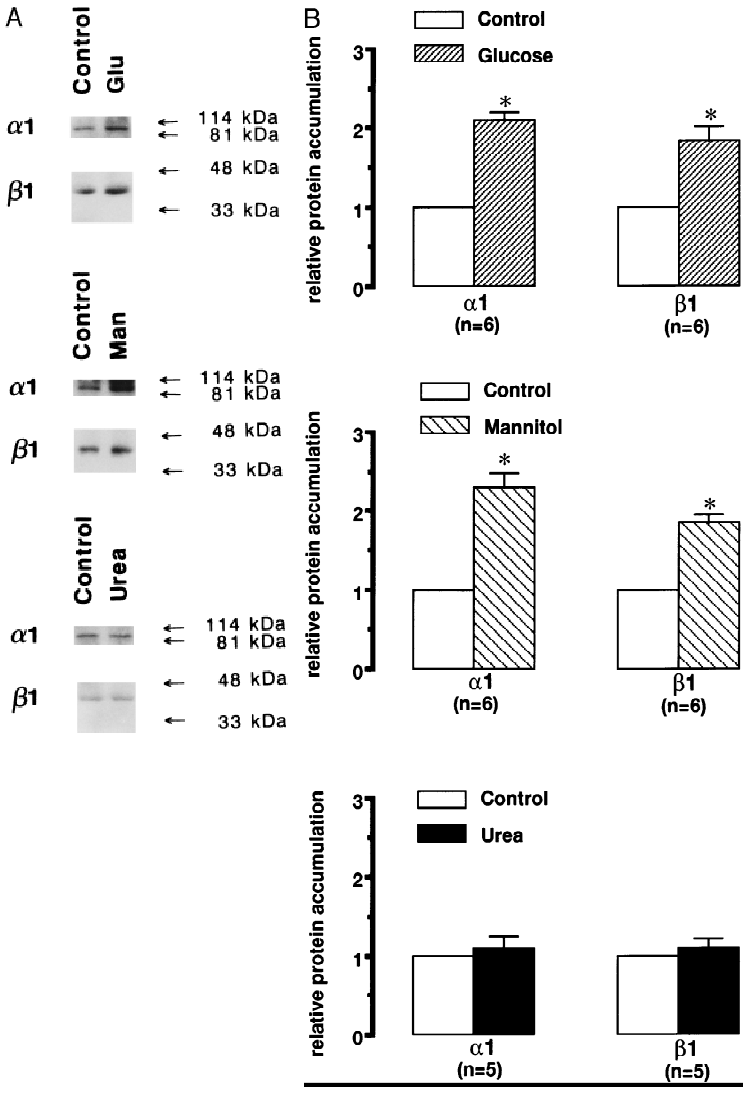


Fig. 9. Effects of hyperosmolality on Na, K-ATPase α1- and β1-subunit protein accumulation. VSMC were exposed to MEM treated with glucose, mannitol, or urea (500 mOsm/kgH_2O) for 24 hr. *A:* Western blot analyses, *B:* quantitations of the Western blots. Abundance of each subunit protein is expressed as units of densitometry relative to *control.* Data are means ± SE. *$P < 0.005$ compared with *control.*

β1-mRNA levels, as described previously by Muto et al. [22]. Similar findings have also been reported in a rat liver cell line [27] in which cycloheximide induced α1- and β1-mRNA accumulation. We propose the possibility that in VSMC, protein(s) which inhibit α1-mRNA expression are present under control isosmotic conditions. However, this possibility will require further investigation. On the other hand, cycloheximide inhibits α1- and β1-mRNA upregulation under hypertonic conditions. These findings indicate that hypertonicity-mediated α1- and β1-mRNA induction occurs, at least in part, through *de novo* synthesis of intermediate regulatory proteins. MAP kinase [16, 34], MAP kinase-kinase [34], and the early gene transcription factors, *Egr*-1 and *c-fos* [7], are all reported to be upregulated in MDCK cells exposed to hypertonic stress. However, in VSMC it is not known whether hypertonicity-mediated α1- and β1-mRNA induction occurs through *de novo* synthesis of these specific proteins.

Actinomycin D blocks DNA transcription and mRNA synthesis by binding to the guanosine residues. Actinomycin D can be used to investigate mRNA turnover, because it does not markedly affect post-transcriptional steps that lead to mRNA degradation. In the presence of actinomycin D, hyperosmotic glucose or mannitol media did not show an appreciable effect on α1- or β1-mRNA accumulation. Therefore, the hypertonicity-induced α1- and β1-mRNA upregulation resulted from an increase in mRNA synthesis at either the transcription or the RNA processing steps, rather than from a decrease in the mRNA degradation rate.

It is generally accepted that, in a wide variety of cell systems [16, 28, 29, 33], elevation of intracellular Na concentration leads to an induction of Na, K-ATPase mRNA and Na, K-ATPase activity. In VSMC, hyperosmolality is reported to cause Na/H exchange [25, 30] and Na-K-2Cl cotransport [25] to activate. These reports raised the possibility that in VSMC, hyperosmolality-

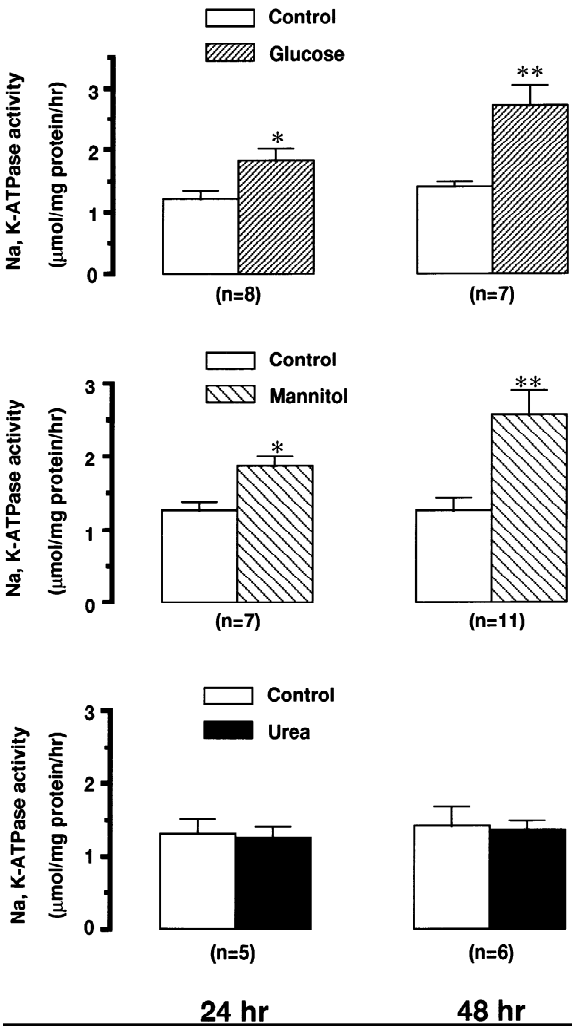


Fig. 10. Effects of hyperosmolality on Na, K-ATPase activity. VSMC were exposed to MEM treated with glucose, mannitol, or urea (500 mOsm/kgH_2O) for 24 or 48 hr. Data are means ± SE. *$P < 0.05$, **$P < 0.005$ compared with *control.*

induced upregulation of α1- and β1-mRNA may be due to early increases in Na influx into the cell and in intracellular Na concentration. However, neither Na/H antiport inhibitor (ethylisopropylamiloride) nor Na-K-2Cl cotransport inhibitor (bumetanide) affected the hyperosmolality-induced upregulation of α1- or β1-mRNA. Furthermore, removal of Na from the medium had no effect on the hyperosmolality-induced upregulation of α1- or β1-mRNA. Accordingly, hyperosmolality-induced upregulation of α1- and β1-mRNA occurs through Na-independent mechanisms. In sharp contrast to VSMC, in renal IMCD cells [24] the early stimulation of Na uptake into the cell is required steps in the hyperosmolality-induced Na, K-ATPase gene expression. The mechanism(s) for this differential effect is not clear, but may reflect a fundamental difference in cell type-specific characteristics.

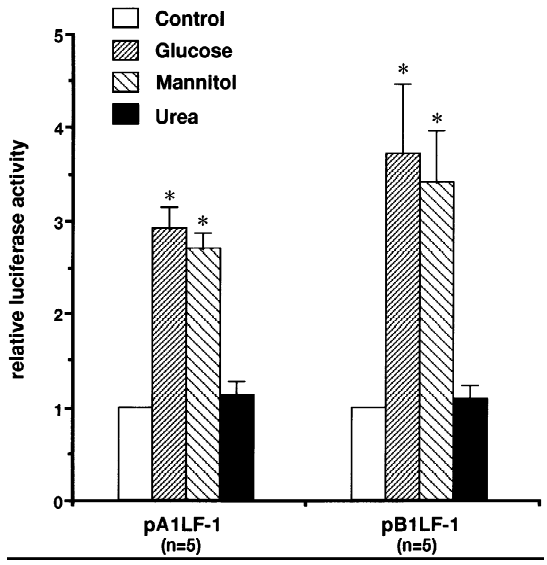


Fig. 11. Effects of hyperosmolality on luciferase activities for the α1- and β1-subunit genes (pA1LF-1 and pB1LF-1, respectively) in VSMC. VSMC were exposed to MEM treated with glucose, mannitol, or urea (500 mOsm/kgH_2O) for 24 hr. Luciferase activities were normalized with cell protein content, and expressed as relative increase compared with *control.* Data are means ± SE. *$P < 0.005$ compared with *control.*

Hyperosmolality is known to activate MAP kinase in yeast [3] and in MDCK cells [16, 34]. This MAP kinase activation occurs in a PKC-dependent fashion [3, 16, 34]. In addition, long-term exposure of hypertonic medium to VSMC is reported to increase PKC activity [30]. To evaluate whether PKC signaling pathways contribute to α1- or β1-mRNA upregulation under hypertonic conditions, we used two inhibitors of PKC. PKC inhibitors (staurosporine A or calphostin C) reduced the levels of hypertonicity-induced β1-mRNA, whereas they caused no effect on the levels of hypertonicity-induced α1-mRNA (Fig. 7). From these observations, we conclude that the hypertonicity-induced β1-mRNA upregulation occurs through PKC signaling pathways, whereas the hypertonicity-induced α1-mRNA upregulation occurs through PKC-independent mechanisms. Hyperosmolality is also known to activate MAP kinase via tyrosine phosphorylation of *HOG1*, which encodes members of the MAP kinase gene families in yeast and in mammalian cells [13]. In addition, hyperosmolality inhibits bicarbonate absorption in rat renal medullary thick ascending limb via a TK-dependent pathway [12]. Therefore, we used two TK inhibitors to examine a possible role of TK in the hypertonicity-induced upregulation of α1- and β1-mRNA. The TK inhibitors used in the present study block TK action differently: genistein competes at the ATP binding site of TK [1], and herbimycin A inhibits TK by binding to reactive sulfhydryl groups of the kinase [36]. We demonstrated that genistein or herbimycin A suppressed the levels of hypertonicity-

mediated β1-mRNA, but not the levels of hypertonicity-mediated α1-mRNA (Fig. 8). From these findings, we conclude that hypertonicity-induced β1-mRNA upregulation occurs through activation of TK, whereas hypertonicity-induced α1-mRNA upregulation occurs independently of TK. Therefore, under hypertonic conditions mechanisms for the α1- and β1-mRNA upregulation are different. Inhibitors of PKC or TK also show different effects on hypertonicity-induced β1-mRNA upregulation: staurosporine A is more potent than calphostin C, and genistein seems stronger than herbimycin A. Although specific inhibitory potential differs among these agents [6], in the present study we did not focus on the differences in specificity among these kinase inhibitors.

To gain some insight into the regulatory mechanisms by which hypertonicity increases Na, K-ATPase mRNA abundance at the transcriptional level, DNA of the 5′-flanking regions of the rat Na, K-ATPase α1- and β1-subunit genes linked to the firefly luciferase reporter gene, was transfected into VSMC. Hypertonic media supplemented with glucose or mannitol significantly increased promoter activities for the α1- and β1-subunit genes (Fig. 11). These results indicate that the hypertonicity-response elements reside between −1,537 and +261 of the α1-subunit gene and between −764 and +129 of the β1-subunit gene. Further studies will be needed to identify and characterize these elements in the Na, K-ATPase α1- and β1-subunit genes.

In summary, we clearly demonstrate that in VSMC, hyperosmolality induced by impermeable solutes (glucose or mannitol) stimulates α1- and β1-mRNA accumulation, α1- and β1-subunit protein accumulation, and Na, K-ATPase activity, whereas hyperosmolality induced by the permeable solute (urea) causes no effect. The stimulatory effect of impermeable solutes on Na, K-ATPase gene expression may help to maintain intracellular Na and K concentrations in VSMC under long-term hyperosmotic conditions.

We would like to thank Ms. Y. Watanabe for her excellent technical assistance and Dr. Y. Murakami for β1-subunit gene reporter construct.

This work was supported by the Science Research Promotion Fund of the Japan Private School Promotion Foundation, by Research Foundation for Community Medicine, the Fund from the Japanese Kidney Foundation (Jinkenkyukai), and Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

References

1. Akiyama, T., Ishida, J., Nakagawa, S., Ogawa, H., Watanabe, S., Itoh, N., Shibuya, M., Fukami, Y. 1987. Genistein, a specific inhibition of tyrosine-specific protein kinases. *J. Biol. Chem.* **262:**5592–5595
2. Bowen, J.W. 1992. Regulation of Na-K-ATPase expression in cultured renal cells by incubation in hypertonic medium. *Am. J. Physiol.* **262:**C845–C852
3. Brewster, J.L., de Valoir, T., Dwyer, N.D., Winter, E., Gustin, M.C. 1993. An osmosensing signal transduction pathway in yeast. *Science* **259:**1760–1763
4. Chamberlin, M.E., Strange, K. 1989. Anisosmotic cell volume regulation: A comparative review. *Am. J. Physiol.* **257:**C159–C173
5. Chen, C., Okayama, H. 1987. High-efficiency transformation of mammalian cells by plasmid cDNA. *Mol. Cell. Biol.* **7:**2745–2752
6. Cohen, D.M., Gullans, S.R., Chin, W.W. 1996. Urea signaling in cultured murine inner medullary collecting duct (mIMCD3) cells involves protein kinase C, inositol 1, 4, 5-triphosphate (IP3), and a putative receptor tyrosine kinase. *J. Clin. Invest.* **97:**1884–1889
7. Cohen, D.M., Wasserman, J.C., Gullans, S.R. 1991. Immediately early gene and HSP 70 expression in hyperosmotic stress in MDCK cells. *Am. J. Physiol.* **261:**C594–C601
8. de Wet, J.R., Wood, K.V., DeLuca, M., Helinski, D.R., Subramani, S. 1987. Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.* **7:**725–737
9. Fleming, W.W. 1980. The electrogenic Na^+, K^+ pump in smooth muscle: physiologic and pharmacologic significance. *Ann. Rev. Pharmacol. Toxicol.* **20:**129–149
10. Garg, L.C., Saha, P.K., Mohuczy-Dominiak, D. 1993. Cholinergic inhibition of Na-K-ATPase via activation of protein kinase C in Madin-Darby canine kidney cells. *J. Am. Soc. Nephrol.* **4:**195–205
11. Gloor, S., Antonicek, H., Sweadner, K.J., Pagliusi, S., Frank, R., Moos, M., Schachner, M. 1990. The adhesion molecule on glia (AMOG) is a homologue of the beta subunit of the Na, K-ATPase. *J. Cell Biol.* **110:**165–170
12. Good, D.W. 1995. Hyperosmolality inhibits bicarbonate absorption in rat medullary thick ascending limb via a protein-tyrosine kinase-dependent pathway. *J. Biol. Chem.* **270:**9833–9889
13. Han, J., Lee, J.-D., Bibbs, L., Ulevitch, R.J. 1994. A MAP-kinase targeted by endotoxin and hyperosmolality in mammalian cells. *Science* **265:**808–811
14. Hara, Y., Urayama, O., Kawakami, K., Nojima, H., Kojima, T., Ohta, T., Nagano, K., Nakao, M. 1987. Primary structures of two types of alpha-subunit of rat brain Na^+-K^+-ATPase deduced from cDNA sequences. *J. Biochem.* **102:**43–58
15. Hoffmann, E.K., Simonsen, L.O. 1989. Membrane mechanisms in volume and pH regulation in vertebrate cells. *Physiol. Rev.* **69:**315–382
16. Itoh, T., Yamauchi, A., Miyai, A., Yokoyama, K., Kamada, T., Ueda, N., Fujiwara, Y. 1994. Mitogen-activated protein kinase and its activator are regulated by hypertonic stress in Madin-Darby canine kidney cells. *J. Clin. Invest.* **93:**2387–2392
17. Kirtane, A., Ismail-Beigi, N., Ismail-Beigi, F. 1994. Role of enhanced Na^+ entry in control of Na, K-ATPase gene expression by serum. *J. Membrane Biol.* **137:**9–15
18. Lingrel, J.B., Orlowski, J., Shull, M.M., Price, E.M. 1990. Molecular genetics of Na, K-ATPase. *Prog. Nucl. Acid Res. Mol. Biol.* **38:**37–89
19. Liu, B., Gick, G. 1992. Characterization of the 5′ flanking region of the rat Na^+/K^+-ATPase β1 subunit gene. *Biochem. Biophys. Acta.* **1130:**336–338
20. Malik, N., Canfield, V.A., Beckers, M.-C., Gros, P., Levenson, R. 1996. Identification of the mammalian Na, K-ATPase β3 subunit. *J. Biol. Chem.* **271:**22754–22758
21. Martin-Vasallo, P., Dackowski, W., Emanuel, J.R., Levenson, R. 1989. Identification of a putative isoform of the Na, K-ATPase β subunit. Primary structure and tissue-specific expression. *J. Biol. Chem.* **264:**4613–4618
22. Muto, S., Nemoto, J., Ohtaka, A., Watanabe, Y., Yamaki, M., Kawakami, K., Nagano, K., Asano, Y. 1996. Differential regulation of Na^+-K^+-ATPase gene expression by corticosteroids in vascular smooth muscle cells. *Am. J. Physiol.* **270:**C731–C739

23. Ohara, T., Ikeda, U., Muto, S., Oguchi, A., Tsuruya, Y., Yamamoto, K., Kawakami, K., Shimada, K., Asano, Y. 1993. Thyroid hormone stimulates Na^+-K^+-ATPase gene expression in cultured rat mesangial cells. *Am. J. Physiol.* **265:**F370–F376
24. Ohtaka, A., Muto, S., Nemoto, J., Kawakami, K., Nagano, K., Asano, Y. 1996. Hyperosmolality stimulates Na-K-ATPase gene expression in inner medullary collecting duct cells. *Am. J. Physiol.* **270:**F728–F738
25. Orlov, S.N., Resink, T.J., Bernhardt, J., Buhler, F.R. 1992. Volume-dependent regulation of sodium and potassium fluxes in cultured vascular smooth muscle cells. *J. Membrane Biol.* **129:**199–210
26. Orlowski, J., Lingrel, J.B. 1988. Tissue-specific and developmental regulation of rat Na^+-K^+-ATPase catalytic α-isoform and β-subunit mRNAs. *J. Biol. Chem.* **263:**10436–10442
27. Perez, C., Bhutada, A., Ismail-Beigi, F. 1991. Induction of Na^+-K^+-ATPase subunit mRNAs by cycloheximide in a rat liver cell line. *Am. J. Physiol.* **260:**C417–C423
28. Pollack, L.R., Tate, E.H., Cook, J.S. 1981. Turnover and regulation of Na-K-ATPase in HeLa cells. *Am. J. Physiol.* **256:**C173–C183
29. Rayson, B.M. 1989. Rates of synthesis and degradation of Na^+-K^+-ATPase during chronic ouabain treatment. *Am. J. Physiol.* **256:**C75–C80
30. Soleimani, M., Singh, G., Dominguez, J.H., Howard, R.L. 1995. Long-term high osmolality activates Na-H exchange and protein kinase C in aortic smooth muscle cells. *Circ. Res.* **76:**530–535
31. Suzuki-Yagawa, Y., Kawakami, K., Nagano, K. 1992. Housekeeping Na, K-ATPase α1 subunit gene promoter is composed of multiple cis elements to which common and cell type-specific factors bind. *Mol. Cell. Biol.* **12:**4046–4055
32. Sweadner, K.J. 1989. Isozymes of the Na^+-K^+-ATPase. *Biochim. Biophys. Acta.* **988:**185–220
33. Taormino, J.P., Fambrough, D.M. 1990. Pre-translational regulation of the Na, K-ATPase in response to demand for ion transport in cultured chicken skeletal muscle. *J. Biol. Chem.* **265:**4116–4123
34. Terada, Y., Tomita, K., Homma, M.K., Nonoguchi, H., Yang, T., Yamada, T., Yuasa, Y., Krebs, E.G., Sasaki, S., Marumo, F. 1994. Sequential activation of Raf-1 kinase, mitogen-activated protein (MAP) kinase, MAP kinase kinase, and S6 kinase by hyperosmolality in renal cells. *J. Biol. Chem.* **269:**31296–31301
35. Uchida, S., Garcia-Perez, A., Murphy, H., Burg, M.B. 1989. Signal for induction of aldose reductase in renal medullary cells by high external NaCl. *Am. J. Physiol.* **256:**C614–C620
36. Uehara, Y., Fukazawa, H. 1991. Use and selectivity of herbimycin A as inhibitor of protein-tyrosine kinase. *Methods Enzymol.* **201:**370–379
37. Yamamoto, K., Ikeda, U., Okada, K., Saito, T., Kawakami, K., Shimada, K. 1994. Sodium ion mediated regulation of Na, K-ATPase gene expression in vascular smooth muscle cells. *Cardiovasc. Res.* **28:**957–962
38. Yordy, M., Bowen, J.W. 1993. Na, K-ATPase expression and cell volume during hypertonic stress in human renal cells. *Kidney Int.* **43:**940–948
39. Young, R.M., Shull, G.E., Lingrel, J.B. 1987. Multiple mRNAs from rat kidneys and brain encoding a single Na^+, K^+-ATPase β subunit protein. *J. Biol. Chem.* **262:**905–4110