

# Effect of Inhibition of $\text{Na}^+/\text{K}^+$ -Adenosine Triphosphatase on Vascular Action of Vasopressin

Koji Okada, Carlos Caramelo, Phoebe Tsai, and Robert W. Schrier

Department of Medicine, University of Colorado School of Medicine, Denver, Colorado 80262

## Abstract

The present study was undertaken to examine the cellular interaction between a  $\text{Na}^+/\text{K}^+$ -ATPase inhibitor, ouabain, and arginine vasopressin (AVP) in rat vascular smooth muscle cells (VSMC) in culture. Preincubation with  $10^{-5}$  M ouabain for 60 min increased basal cytosolic free  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) concentration and intracellular  $^{45}\text{Ca}^{2+}$  uptake. Ouabain, however, did not affect basal  $^{45}\text{Ca}^{2+}$  efflux or AVP-stimulated  $^{45}\text{Ca}^{2+}$  efflux. As assessed by cell shape change, preincubation with  $10^{-5}$  M ouabain for 60 min also enhanced the sustained cellular contractile effect of a submaximal ( $10^{-8}$  M AVP, 21.5% vs. 30.5%,  $P < 0.01$ ) but not maximal dose of  $10^{-6}$  M AVP. Preincubation with  $10^{-5}$  M ouabain for 60 min did not change AVP-induced  $\text{V}_1$ -specific surface receptor binding or AVP-induced inositol phosphate production but did however potentiate the mobilization of  $[\text{Ca}^{2+}]_i$  induced by a submaximal ( $10^{-8}$  M AVP, 301 vs. 385 nM,  $P < 0.01$ ) but not a maximal dose of AVP. These effects of ouabain on the mobilization of  $[\text{Ca}^{2+}]_i$  were abolished by incubation in  $\text{Ca}^{2+}$ -free buffer or  $5 \times 10^{-5}$  M verapamil. Ouabain ( $10^{-5}$  M) also enhanced the sustained cellular contractile effect of a direct protein kinase C activator, phorbol 12-myristate 13-acetate. The present results therefore indicate that the inhibition of  $\text{Na}^+/\text{K}^+$ -ATPase may enhance the vascular action of AVP, and perhaps other vasoconstrictors, by increasing the AVP-induced mobilization of  $[\text{Ca}^{2+}]_i$  and by potentiating the activity of protein kinase C stimulated by AVP through enhancing basal and AVP-stimulated cellular  $\text{Ca}^{2+}$  uptake. (*J. Clin. Invest.* 1990. 86:1241–1248.) Key words: arginine vasopressin • calcium •  $\text{Na}^+/\text{K}^+$ -adenosine triphosphatase • ouabain • vascular smooth muscle cell

## Introduction

A current theory on the pathogenesis of arterial hypertension proposes that an increased intracellular  $\text{Na}^+$  concentration in the smooth muscle cells of the arterial wall is an important event in the generation of hypercontractility of the resistance vessels (1, 2). Such an increase in intracellular  $\text{Na}^+$  appears to provoke increased  $\text{Na}^+/\text{Ca}^{2+}$  exchange, leading to the elevation of intracellular  $\text{Ca}^{2+}$ , which in turn favors smooth muscle cell contraction and increased arterial pressure (1–7). In recent years, circulating digitalis-like substances, with  $\text{Na}^+/\text{K}^+$ -ATP-

ase inhibitory activity, have been proposed as principal mediators of increased intracellular  $\text{Na}^+$  concentration in hypertension (1, 2). An increased digitalis-like activity has been detected in hypertensive humans (8–14) as well as in animal models of hypertension (15–19). The  $\text{Na}^+/\text{K}^+$ -ATPase inhibitors have been also reported to enhance the action of vasopressor hormones (2, 5, 20, 21). However, in spite of evidence indicating that the  $\text{Na}^+/\text{K}^+$ -ATPase inhibitors increase vascular contractility and enhance the action of vasopressor hormones, the intracellular mechanisms whereby  $\text{Na}^+/\text{K}^+$ -ATPase inhibition enhances the cellular action of vasopressor hormones is not known (22).

The present study was therefore undertaken to examine the cellular interaction of a  $\text{Na}^+/\text{K}^+$ -ATPase inhibitor, ouabain, and a vasopressor hormone, arginine vasopressin (AVP), in rat vascular smooth muscle cells (VSMC)<sup>1</sup> in culture. The effect of ouabain on the cellular action of AVP was studied by using cell shape change as an index of the sustained cellular contractile response. The cellular interactions between ouabain and AVP were investigated by examining the effects of ouabain on (a) AVP  $\text{V}_1$  receptor binding, (b) AVP-induced inositol phosphate production, (c) basal and AVP-stimulated intracellular  $^{45}\text{Ca}^{2+}$  uptake, (d) basal and AVP-induced  $^{45}\text{Ca}^{2+}$  efflux, and (e) basal and AVP-mobilized cytosolic free  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ). In addition, the effects of the calcium channel blocker, verapamil, and the removal of extracellular  $\text{Ca}^{2+}$  were also examined to assess the mechanism of the effect of ouabain on the cellular action of AVP. Finally, the effect of ouabain on the sustained cellular contraction produced by the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA) was also examined.

## Methods

**Cell culture.** Rat VSMC were isolated using a modification (23–25) of the technique described by Chamley et al. (26). Briefly, the rat thoracic aortas dissected from 8–10 Sprague-Dawley male rats (250–300 g) were incubated in Eagle's minimum essential medium (MEM) containing 2 mg/ml collagenase (Cooper Biomedical Inc., Malvern, PA) for 60 min at 37°C. After the removal of adventitia and small fragments of the outer membrane, the aortas were minced and incubated in Eagle's MEM containing 2 mg/ml collagenase for 2 h at 37°C. The freshly isolated cells were resuspended in Eagle's MEM containing 1  $\mu\text{M}$  L-glutamine, 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 10% fetal calf serum, pH 7.4. The cells were plated in 35  $\times$  10-mm culture dishes at a density of  $1 \times 10^6$  and  $2 \times 10^6$  cells/ml for the cell contraction studies and the subculture, respectively. The cells were incubated in a humidified atmosphere of 95% air and 5%  $\text{CO}_2$  at 37°C. All of the experiments except the cell contraction studies were performed in subcultured cells within the fourth passage. The subculture was performed by trypsin-EDTA (0.25–0.1%) treatment. The identity

Address reprint requests to Dr. Robert W. Schrier, C281, University of Colorado School of Medicine, 4200 East 9th Avenue, Denver, CO 80262.

Received for publication 7 September 1988 and in revised form 12 February 1990.

J. Clin. Invest.

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0021-9738/90/10/1241/08 \$2.00

Volume 86, October 1990, 1241–1248

1. Abbreviations used in this paper:  $\text{IP}_2$ , inositol bisphosphate;  $\text{IP}_3$ , inositol trisphosphate; PSS, physiological saline solution; VSMC, vascular smooth muscle cells.

of cells was confirmed by electron microscopy and viability was > 95%, as judged by Trypan blue exclusion.

**Cell contraction studies.** The sustained cellular contractile response was assessed by quantitation of cell surface area changes using phase-contrast microscopy (model IM, Carl Zeiss, Oberkochen, FRG) and a digital imaging analysis system (Zidas, Carl Zeiss). This morphometric analysis is similar to the method reported from our and other laboratories (23, 25, 27). The cell contraction studies were performed 6–8 d after seeding the primary cultures. The magnitude of the shape change response was determined by comparing groups of 14–20 cells. The measurements of cell surface areas were obtained in triplicate and the mean value of one cell was used for comparison. The cells were washed twice with 2 ml physiological saline solution (PSS) (140 mM NaCl, 4.6 mM KCl, 1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 10 mM glucose, and 10 mM Hepes, pH 7.4) before the first measurement of cell surface area. The cells were incubated with 2 ml of PSS or 10<sup>-5</sup> M ouabain (Sigma Chemical Co., St. Louis, MO) for 60 min at 37°C and the second measurement of cell surface area was performed. The third measurement of cell surface area was done after 20 min incubation at 37°C with 2 ml PSS or 10<sup>-5</sup> M ouabain in the absence or presence of effectors; specifically, AVP or PMA (Sigma Chemical Co.) to examine the effects of ouabain on the AVP- or PMA-induced sustained cellular contractile response. The cells with more than a 10% decrease in cell surface area between the first and the second measurement or between the second and the third measurement were considered the contractile cells. The results are expressed as a percentage of the contractile cells.

**AVP V<sub>1</sub> receptor binding study.** Confluent cell monolayers grown on 35 × 10-mm plastic dishes were used to examine the effect of ouabain on AVP V<sub>1</sub> receptor binding. After the cells were washed twice with 2 ml of PSS, the cells were incubated with 2 ml of PSS or 10<sup>-5</sup> M ouabain for 60 min at 37°C. At the end of the incubation period the cells were washed twice with 2 ml of ice-cold PSS or 10<sup>-5</sup> M ouabain and then incubated with 2 ml of ice-cold PSS or 10<sup>-5</sup> M ouabain containing 2 × 10<sup>-9</sup> M [<sup>3</sup>H]AVP (specific activity, 67.7 Ci/mmol, New England Nuclear, Boston, MA) in the absence or presence of 1 × 10<sup>-6</sup> M AVP at 4°C for 60 min. After the incubation the cells were rinsed four times with 2 ml of ice-cold PSS and dissolved with 2 ml of sodium dodecyl sulfate (SDS)-alkaline solution (0.1% SDS and 0.1 N NaOH). Samples (100 μl) were stored at 4°C for protein assay by the method of Lowry et al. (28) and the radioactivity of SDS-alkaline solution was counted using a liquid scintillation counter (Tri-Carb 460C, Packard Instrument Co., Inc., Downers Grove, IL).

**Measurement of inositol phosphates.** Confluent monolayers on 35 × 10-mm plastic dishes were also used in this study. The measurement of the intracellular levels of inositol phosphates was performed as described (24, 25). The cells were washed twice with 2 ml of inositol-free Dulbecco's modified Eagle's MEM (DME) (Hazleton Research Products Inc., Lenexa, KS) and incubated for 2 d with 1.5 ml of inositol-free DME containing 5 μCi/ml myo-2 [<sup>3</sup>H]inositol (specific activity, 19 Ci/mmol, Amersham Corp., Arlington Heights, IL) in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C. At the time of the experiment, the cells were washed twice with 2 ml of PSS and then incubated with 1 ml of PSS or 10<sup>-5</sup> M ouabain for 60 min at 37°C. At the end of the incubation the cells were exposed for 10 s at 37°C to 1 ml of PSS or 10<sup>-5</sup> M ouabain in the absence or presence of 10<sup>-8</sup> or 10<sup>-6</sup> M AVP. The reaction was stopped by the addition of 100 μl of ice-cold 100% trichloroacetic acid and the cells were scraped using an Eppendorf pipette tip. The suspensions containing the disrupted cells were centrifuged using a microcentrifuge (model 5413, Eppendorf, Brinkmann Instruments Co., Westbury, NY). The supernatants were washed four times with 3 ml of ether and the water-soluble fractions were brought to pH 7.0 using 1 M Tris-base and stored at -20°C until analysis. The pellets were dissolved with 1 ml SDS-alkaline solution and stored at 4°C for protein assay. The water-soluble fractions were applied to columns containing 1 ml of Dowex (1-x8, formate form) and serially eluted with 2-ml aliquots of H<sub>2</sub>O, Borax (5 mM disodium tetraborate [Sigma Chemical Co.], 60 mM sodium formate [Sigma Chemical

Co.]), 0.2, 0.4, and 1.0 M ammonium formate (Sigma Chemical Co.) in 0.1 M formic acid, separating inositol, glycerophosphatidylinositol, inositol-1-phosphate, inositol bisphosphate (IP<sub>2</sub>), and inositol trisphosphate (IP<sub>3</sub>), respectively. IP<sub>3</sub> fraction includes 1,3,4- and 1,4,5-IP<sub>3</sub> and 1,3,4,5-inositol phosphate. Samples were collected in scintillation counter vials and counted using a liquid scintillation counter. The extraction procedure has been previously validated in this laboratory with radioactive phosphoinositides.

**Intracellular <sup>45</sup>Ca<sup>2+</sup> uptake.** Confluent monolayers of cells on 60 × 15-mm plastic culture dishes were used to measure intracellular <sup>45</sup>Ca<sup>2+</sup> uptake. The cells were washed twice with 2 ml of PSS and then incubated with 2 ml of PSS or 10<sup>-5</sup> M ouabain containing 2 μCi/ml <sup>45</sup>Ca<sup>2+</sup> (specific activity, 12.3 mCi/mg Ca<sup>2+</sup>; ICN Radiochemicals, Irvine, CA) at 37°C for different times (1, 5, 10, 15, 30, and 60 min). After the incubation period, external <sup>45</sup>Ca<sup>2+</sup> was removed by rinsing the dish three times with 5 ml of ice-cold Ca<sup>2+</sup>-free PSS containing 1 mM EGTA. The cells were treated with hypotonic shock by the addition of 1 ml of deionized water and the disrupted cells were collected using a rubber policeman. The suspension containing the disrupted cells was placed into a microcentrifuge tube with 0.25 ml 4 N perchloric acid and centrifuged using a microcentrifuge (model 5413, Eppendorf). The radioactivity of supernatants and the protein of pellets were measured using the same methods as previously described.

**<sup>45</sup>Ca<sup>2+</sup> efflux study.** Confluent monolayers of cells on 35 × 10-mm plastic dishes were used in this study. The cells were washed twice with 2 ml of PSS and preincubated for 60 min at 37°C with 2 ml of PSS or 10<sup>-5</sup> M ouabain containing 8 μCi/ml <sup>45</sup>Ca<sup>2+</sup> (ICN Radiochemicals). At the end of the preincubation the cells were rapidly washed 10 times during 60 s with 2 ml of PSS or 10<sup>-5</sup> M ouabain. The medium was removed and replaced with 2 ml of PSS or 10<sup>-5</sup> M ouabain at each minute from 1 to 6 min. AVP was added at 7 min of the time course of <sup>45</sup>Ca<sup>2+</sup> efflux and 1-min samples collected for an additional 5 min. The amount of <sup>45</sup>Ca<sup>2+</sup> lost from the cells in each time interval was measured by a liquid scintillation counter. The cells were dissolved for the measurement of intracellular radioactivity and the protein content.

**Measurement of [Ca<sup>2+</sup>]<sub>i</sub> concentration.** Confluent monolayers of cells were grown on round glass coverslips (13 mm diam) (Fisher Scientific Co., Pittsburgh, PA) for the measurement of [Ca<sup>2+</sup>]<sub>i</sub>. The fluorescence indicator dye, fura2/AM (Molecular Probes, Inc., Eugene, OR), was used. After the cells were washed twice with PSS, the cells were incubated with PSS or 10<sup>-5</sup> M ouabain containing 4 μM fura2/AM at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> for 60 min. At the end of the loading period the cells were washed twice with PSS or 10<sup>-5</sup> M ouabain and inserted into quartz cuvettes (Fisher Scientific Co.) with 3 ml of PSS or 10<sup>-5</sup> M ouabain. AVP was added to the cells after the measurement of basal [Ca<sup>2+</sup>]<sub>i</sub> concentration. The fluorescent intensity of fura2-loaded cells was measured using a fluorescence spectrophotometer (model 650-10S, Perkin-Elmer Corp., Norwalk, CN) equipped with a thermostatically controlled cuvette holder. The complete intracellular hydrolysis of fura2/AM to fura2 was judged by changes in the excitation and emission spectra. The fluorescence of fura2 was measured using two excitation wavelengths, 345 and 380 nm (5-nm slit), and a 500 nm (5-nm slit) emission wavelength. The ratios of the emitted fluorescence signals at two excitation wavelengths permitted the calculation of [Ca<sup>2+</sup>]<sub>i</sub> concentration independent of cell number, dye loading conditions and dye bleaching. The autofluorescence was measured in similar cells which had not been loaded with fura2/AM and was below 10% of the total fluorescence of fura2 loaded cells. After the subtraction of autofluorescence for each wavelength the [Ca<sup>2+</sup>]<sub>i</sub> concentration was calculated using the method of Grynkiewicz et al. (29). R<sub>max</sub> and R<sub>min</sub> were determined by treating the cells with 5 × 10<sup>-5</sup> M digitonin and 1 × 10<sup>-2</sup> M EGTA, respectively. Since there exists a linear correlation between the maximal change in [Ca<sup>2+</sup>]<sub>i</sub> concentration and the area under the response curve from 0 to 180 s after the addition of the hormonal mediators, maximal changes in [Ca<sup>2+</sup>]<sub>i</sub> were used for the comparison.

**Statistical analysis.** All results were expressed as the mean ± SEM.

The unpaired Student's *t* test was used for statistical comparison. A *P* value of less than 0.05 was considered significant.

## Results

**Effect of ouabain on basal  $[Ca^{2+}]_i$  concentration.** Since the intracellular  $Ca^{2+}$  is thought to be a major determinant of the cellular contractile response to vasopressor hormones, the effect of ouabain on basal  $[Ca^{2+}]_i$  concentration was examined to determine the optimal concentration of ouabain for studying the cellular interaction between ouabain and AVP. The incubation of the cells with the different concentrations of ouabain at 37°C for 60 min increased basal  $[Ca^{2+}]_i$  concentration in a dose-dependent manner (Fig. 1). Ouabain (more than  $10^{-4}$  M) increased basal  $[Ca^{2+}]_i$  concentration beyond 300 nM, which was higher than the maximal  $[Ca^{2+}]_i$  response to  $10^{-8}$  M AVP (see Fig. 6 a). Therefore in the following studies,  $10^{-5}$  M ouabain was used to examine the cellular interaction between ouabain and AVP.

**Effect of ouabain on  $^{45}Ca^{2+}$  uptake and efflux.** Intracellular  $^{45}Ca^{2+}$  uptake and  $^{45}Ca^{2+}$  efflux were measured to examine the mechanisms whereby  $10^{-5}$  M ouabain increases intracellular  $Ca^{2+}$  concentration. In Fig. 2 is shown the time course of the effect of  $10^{-5}$  M ouabain on intracellular  $^{45}Ca^{2+}$  uptake. Ouabain ( $10^{-5}$  M) significantly increased intracellular  $^{45}Ca^{2+}$  uptake beginning 15 min after its addition as compared with that of vehicle. On the other hand, the preincubation of the cells with  $10^{-5}$  M ouabain at 37°C for 60 min did not effect the basal- or AVP-stimulated  $^{45}Ca^{2+}$  efflux (Table I). These results

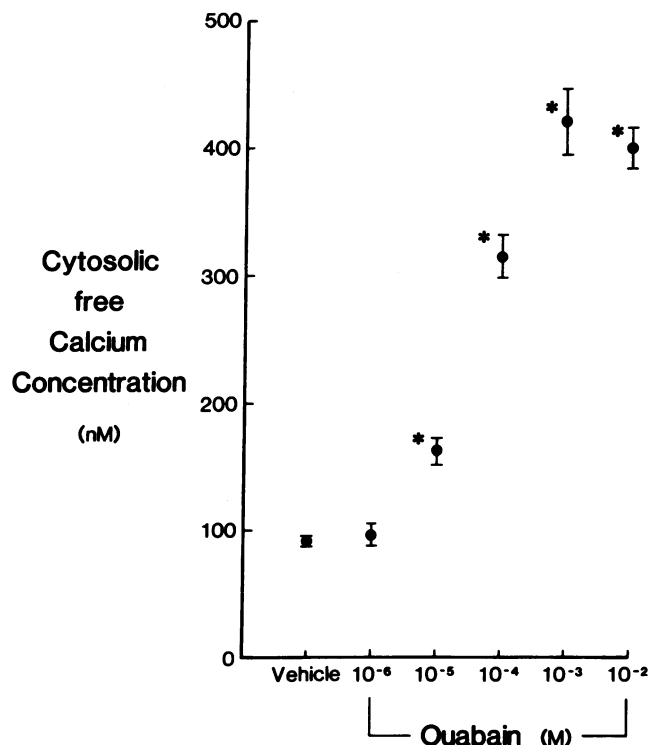


Figure 1. Dose-dependent effect of ouabain on basal cytosolic free  $Ca^{2+}$  ( $[Ca^{2+}]_i$ ) concentration in rat VSMC in culture. The cells were incubated with different concentrations of ouabain for 60 min. Values are expressed as mean  $\pm$  SEM (*n* = 6). \**P* < 0.01 (vs. vehicle).

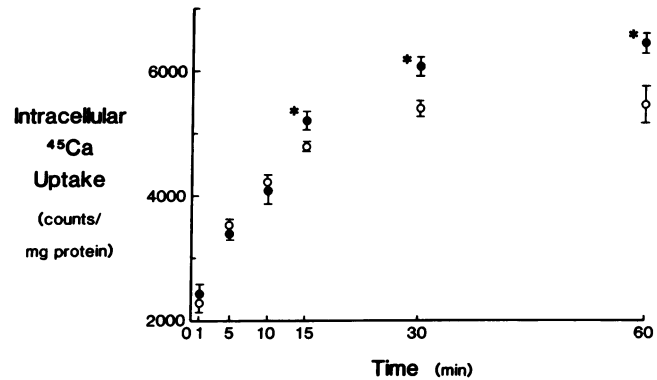


Figure 2. Time course of effect of ouabain on intracellular  $^{45}Ca^{2+}$  uptake in rat VSMC in culture. (○) Vehicle and (●)  $10^{-5}$  M ouabain. Values are expressed as mean  $\pm$  SEM (*n* = 5). \**P* < 0.01 (vs. vehicle).

suggest that  $10^{-5}$  M ouabain increased intracellular  $Ca^{2+}$  concentration by enhancing cellular  $Ca^{2+}$  uptake.

**Effect of ouabain on AVP-induced VSMC contraction.** The effect of ouabain on the cellular action of AVP was studied by using cell shape change as an index of the sustained cellular contractile response. The incubation of the cells with  $10^{-5}$  M ouabain at 37°C for 60 min did not affect the cell contraction as compared with the vehicle. However, preincubation with  $10^{-5}$  M ouabain for 60 min potentiated the AVP-induced sustained cellular contractile response (Fig. 3). AVP induced a sustained (20 min) cellular contractile response in a dose-dependent manner and the maximal cellular contractile response occurred in  $10^{-6}$  M AVP. The potentiating effect of ouabain was evident with submaximal but not maximal doses of AVP. This effect of ouabain was completely blocked by  $5 \times 10^{-5}$  M verapamil (Fig. 4).

**Effect of ouabain on AVP  $V_1$  receptor binding.** Scatchard analysis, obtained by the incubation of the cells with  $2 \times 10^{-9}$  M [ $^3H$ ]AVP for 60 min at 4°C, revealed a single class of surface binding sites with a  $K_d$  of  $4.7 \times 10^{-9}$  mol and  $B_{max}$  of  $1.7 \times 10^{-10}$  mol (30). The preincubation of the cells with  $10^{-5}$  M ouabain at 37°C for 60 min did not change AVP  $V_1$ -specific surface receptor binding ( $100.0 \pm 2.3$  vs.  $95.7 \pm 0.8\%$ , *n* = 3, NS).

**Effect of ouabain on AVP-induced inositol phosphate production.** The preincubation of the cells with  $10^{-5}$  M ouabain at 37°C for 60 min did not affect submaximal ( $10^{-8}$  M) or maximal ( $10^{-6}$  M) AVP-induced  $IP_2$  and  $IP_3$  production (Fig. 5).

**Effect of ouabain on basal and AVP-mobilized  $[Ca^{2+}]_i$ .** AVP mobilized  $[Ca^{2+}]_i$  from the intracellular  $Ca^{2+}$  stores in a dose- and time-dependent manner (Figs. 6, a and b). The preincubation of the cells with  $10^{-5}$  M ouabain at 37°C for 60 min significantly increased basal  $[Ca^{2+}]_i$  and the mobilization of  $[Ca^{2+}]_i$  in response to the submaximal dose of AVP ( $10^{-8}$  M) (Fig. 6 a). The  $[Ca^{2+}]_i$  level 2 min after the addition of  $10^{-8}$  M AVP was also significantly higher in the ouabain-treated cells than that in the vehicle (Fig. 6 a). However,  $10^{-5}$  M ouabain did not enhance the effect of  $10^{-6}$  M AVP to mobilize  $[Ca^{2+}]_i$  (Fig. 6 b). The enhancing effect of ouabain on the AVP ( $10^{-8}$  M)-induced mobilization of  $[Ca^{2+}]_i$  was mainly dependent on an increase in cellular  $Ca^{2+}$  uptake in response to AVP. Since incubation in  $Ca^{2+}$  free media decreased the AVP-induced mobilization of  $[Ca^{2+}]_i$  and also abolished the potentiating ef-

Table I. Absence of Effect of Ouabain on Basal and AVP-induced  $^{45}\text{Ca}^{2+}$  Efflux in Rat VSMC in Culture

Time (min)	Basal			AVP ( $10^{-8}$ M)			AVP ( $10^{-6}$ M)		
	1	2	3	1	2	3	1	2	3
Vehicle (cpm/mg protein)	1,099±52	1,029±40	988±58	4,393±371	6,059±354	2,830±140	8,658±88	5,448±144	2,353±205
Ouabain ( $10^{-5}$ M) (cpm/mg protein)	1,162±31	1,068±30	996±53	4,919±382	6,144±355	3,072±236	8,821±223	5,596±225	2,361±124

Values are expressed as mean±SEM ( $n = 4$ ). Time shows the time after the addition of effectors. There was no statistical difference between the vehicle and ouabain experiments ( $10^{-5}$  M) during the basal or AVP ( $10^{-8}$  or  $10^{-6}$  M) periods.

fect of ouabain on the AVP-mobilized  $[\text{Ca}^{2+}]_i$  without any changes in the effect of ouabain on basal  $[\text{Ca}^{2+}]_i$  (Table II). The effects of ouabain on basal  $[\text{Ca}^{2+}]_i$  concentration and the AVP-induced mobilization of  $[\text{Ca}^{2+}]_i$  were also blocked by  $5 \times 10^{-5}$  M verapamil. As shown in Fig. 7, the incubation of the cells with  $5 \times 10^{-5}$  M verapamil at  $37^\circ\text{C}$  for 60 min induced a significant decrease of basal  $[\text{Ca}^{2+}]_i$  concentration and the maximal change in the  $10^{-8}$  M AVP-induced mobilization of  $[\text{Ca}^{2+}]_i$  as compared with the vehicle. In addition, the preincubation of the cells with  $5 \times 10^{-5}$  M verapamil completely abolished the potentiating effects of ouabain on basal  $[\text{Ca}^{2+}]_i$  concentration and the maximal change in  $[\text{Ca}^{2+}]_i$  in response to  $10^{-8}$  M AVP (Fig. 7).

**Effect of ouabain on PMA-induced VSMC contraction.** In Fig. 8 is shown the potentiating effect of ouabain on the PMA-induced sustained cellular contractile response. PMA has been shown previously to induce a sustained cellular con-

tractile response in a dose- and time-dependent manner without a rise in  $[\text{Ca}^{2+}]_i$  (31). In the present study, the preincubation of the cells with  $10^{-5}$  M ouabain at  $37^\circ\text{C}$  for 60 min significantly potentiated the submaximal PMA-induced contractile response but did not alter the response to a maximal dose of PMA.

## Discussion

Inhibition of  $\text{Na}^+/\text{K}^+$ -ATPase in vascular smooth muscle is proposed to be associated with an increase in cellular  $\text{Na}^+$  concentration, enhanced  $\text{Na}^+/\text{Ca}^{2+}$  exchange and a rise in cellular  $\text{Ca}^{2+}$  concentration (1–7). A rise in  $[\text{Ca}^{2+}]_i$  concentration then may directly increase vascular smooth muscle tone and/or enhance the effect of circulating vasopressor hormones (2, 5, 20, 21). The present study was undertaken to examine the mechanisms whereby an increase in cellular  $\text{Ca}^{2+}$  concentration due to the inhibition of  $\text{Na}^+/\text{K}^+$ -ATPase results in increased contractility of vascular smooth muscle in response to AVP.

It was first necessary to establish the optimal concentration of ouabain suitable for examination of the cellular interaction between ouabain and AVP in cultured rat VSMC. Since preincubation of VSMC with  $> 10^{-4}$  M ouabain for 60 min at  $37^\circ\text{C}$  increased basal  $[\text{Ca}^{2+}]_i$  concentration to a level higher than that induced by  $10^{-8}$  M AVP,  $10^{-5}$  M ouabain was used

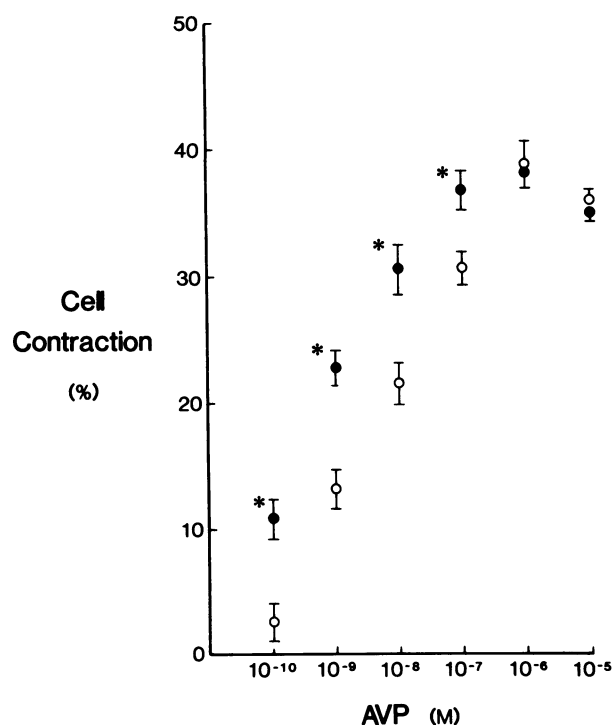


Figure 3. Potentiating effect of ouabain on AVP-induced sustained cellular contractile response in rat VSMC in culture. (○) Vehicle and (●)  $10^{-5}$  M ouabain. Values are expressed as mean±SEM ( $n = 6$ ). \* $P < 0.01$  (vs. vehicle).

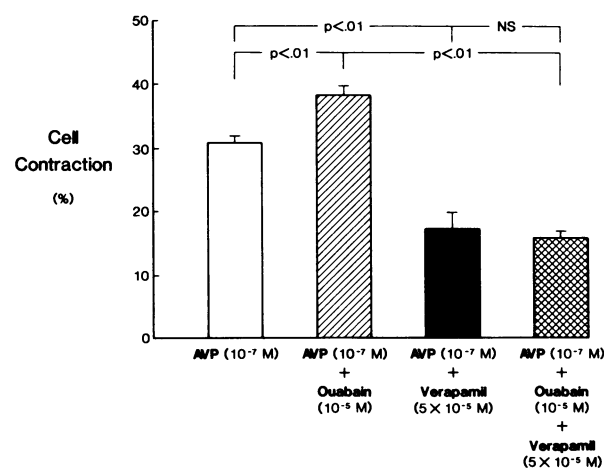


Figure 4. Inhibitory effect of verapamil on potentiation of ouabain in AVP-induced sustained cellular contractile response in rat VSMC in culture. Values are expressed as mean±SEM ( $n = 6$ ).

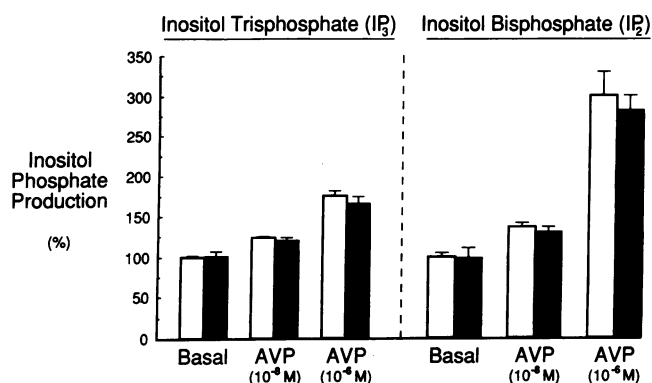


Figure 5. Effect of ouabain on AVP-induced production of inositol phosphates in rat VSMC in culture. Vehicle (open bars) and 10<sup>-5</sup> M ouabain (solid bars). Values are expressed as mean±SEM (n = 5).

in the present studies. In other tissues and VSMC a similar rise in [Ca<sup>2+</sup>]<sub>i</sub> with inhibition of Na<sup>+</sup>/K<sup>+</sup>-ATPase has been suggested to be due to an increase in cellular Ca<sup>2+</sup> uptake (3–8). This interpretation is compatible with the findings in the present studies of a time-dependent enhancement in intracellular <sup>45</sup>Ca<sup>2+</sup> uptake and no change in basal <sup>45</sup>Ca<sup>2+</sup> efflux in VSMC preincubated with 10<sup>-5</sup> M ouabain for 60 min at 37°C. Moreover, either the calcium channel blocker, verapamil, or removal of extracellular Ca<sup>2+</sup> abolished the increase in [Ca<sup>2+</sup>]<sub>i</sub> induced by ouabain.

The present results also demonstrated that preincubation of VSMC with 10<sup>-5</sup> M ouabain enhanced the effect of submaximal, but not maximal, doses of AVP to induce the sustained cellular contractile response. This effect of ouabain was also completely blocked by verapamil. Taken together, these results implicate increased cellular Ca<sup>2+</sup> uptake as a factor in the effect of ouabain to enhance AVP-induced vascular smooth muscle contraction.

Previous studies have shown that after AVP binding to the V<sub>1</sub> receptor, AVP activates phospholipase C to produce (a) IP<sub>3</sub>, which mobilizes [Ca<sup>2+</sup>]<sub>i</sub> from intracellular Ca<sup>2+</sup> stores to induce the initial cellular contractile response (24, 25, 32–36), and (b) diacylglycerol (DAG), (37) which activates protein kinase C (38) to produce the sustained cellular contractile response (31, 39, 40). The cellular interaction between ouabain and AVP was therefore studied by examining the effects of ouabain on AVP V<sub>1</sub> receptor binding, IP<sub>3</sub> production, and

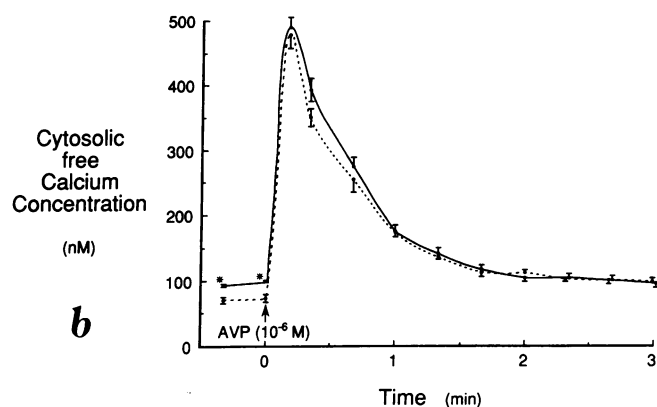
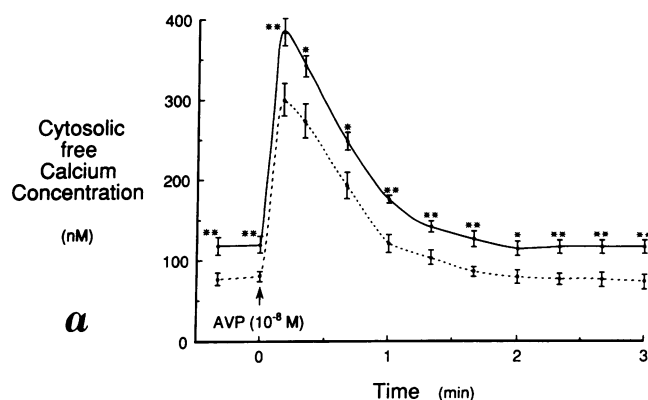


Figure 6. (a) Potentiating effect of ouabain on mobilization of [Ca<sup>2+</sup>]<sub>i</sub> in response to submaximal dose of AVP in rat VSMC in culture. AVP (10<sup>-8</sup> M) was added at 0 min. (○) Vehicle and (●) 10<sup>-5</sup> M ouabain. Values are expressed as mean±SEM (n = 5). \*P < 0.05, \*\*P < 0.01 (vs. vehicle). (b) Effect of ouabain on mobilization of [Ca<sup>2+</sup>]<sub>i</sub> in response to maximal dose of AVP in rat VSMC in culture. AVP (10<sup>-6</sup> M) was added at 0 min. (○) Vehicle and (●) 10<sup>-5</sup> M ouabain. Values are expressed as mean±SEM (n = 5). \*P < 0.01 (vs. vehicle).

mobilization of [Ca<sup>2+</sup>]<sub>i</sub>. The effect of ouabain on the contractile response to the protein kinase C activator, PMA, was also examined to investigate the possible role of a protein kinase C-mediated mechanism in the effect of ouabain on the cellular action of AVP.

Since AVP V<sub>1</sub> receptor binding and AVP-stimulated IP<sub>3</sub> production were not affected by the preincubation with oua-

Table II. Effect of [Ca<sup>2+</sup>]<sub>e</sub> Concentration on an Increase in Basal [Ca<sup>2+</sup>]<sub>i</sub> Concentration and Maximal Change in AVP-induced Mobilization of [Ca<sup>2+</sup>]<sub>i</sub> Due to Ouabain in Rat VSMC in Culture

[Ca <sup>2+</sup> ] <sub>e</sub>	Basal [Ca <sup>2+</sup> ] <sub>i</sub> concentration			Maximal changes in 10 <sup>-8</sup> M AVP-induced mobilization of [Ca <sup>2+</sup> ] <sub>i</sub>		
	Vehicle	10 <sup>-5</sup> M ouabain	P value	Vehicle	10 <sup>-5</sup> M ouabain	P value
	nM			nM		
2 mM	94.0±7.4	169.7±18.9	<0.01	326.0±13.4	459.3±18.1	<0.01
0 mM	35.5±2.1	45.9±5.5	NS	140.6±21.1	135.4±25.4	NS
P value	<0.01	<0.01		<0.01	<0.01	

Values are expressed as mean±SEM (n = 6). Abbreviation: [Ca<sup>2+</sup>]<sub>e</sub>, extracellular Ca<sup>2+</sup> concentration.

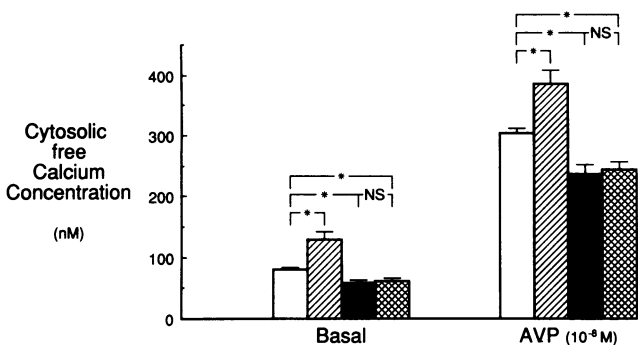


Figure 7. Inhibitory effect of verapamil on potentiation of ouabain in basal  $[Ca^{2+}]_i$  concentration and mobilization of  $[Ca^{2+}]_i$  in response to AVP in rat VSMC in culture. The results represent the maximal changes in  $[Ca^{2+}]_i$ , the vehicle (open bars),  $10^{-5}$  M ouabain (hatched bars),  $5 \times 10^{-5}$  M verapamil (solid bars), and  $10^{-5}$  M ouabain and  $5 \times 10^{-5}$  M verapamil (cross-hatched bars). Values are expressed as mean  $\pm$  SEM ( $n = 6$ ).

bain, direct effects on receptor activation and transmembrane signal transduction did not seem to be involved in the cellular interaction between ouabain and AVP. The preincubation with  $10^{-5}$  M ouabain did, however, enhance the effect of a submaximal, but not maximal, dose of AVP ( $10^{-8}$  M) to increase  $[Ca^{2+}]_i$ . These findings indicate that the ouabain enhancement of AVP-induced vascular smooth muscle contraction is associated with increased  $[Ca^{2+}]_i$  in the absence of alterations in either  $V_1$  receptor binding or phospholipase C

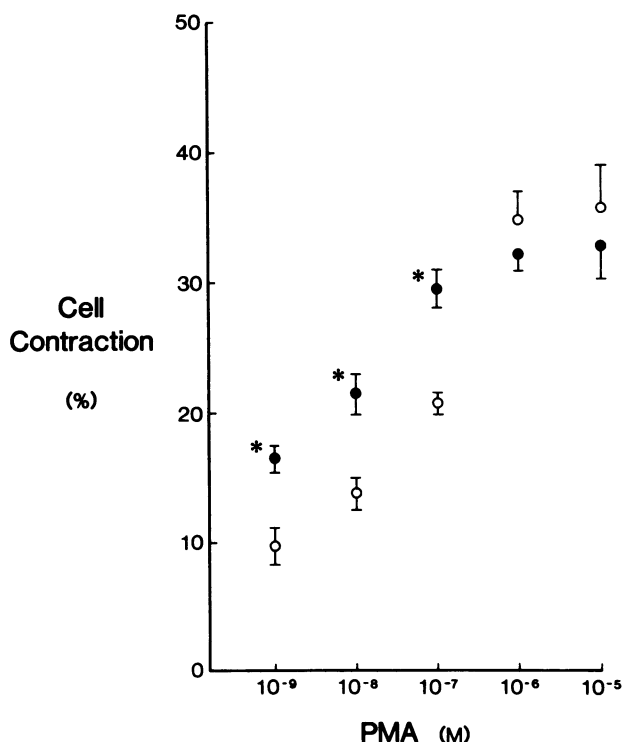


Figure 8. Potentiating effect of ouabain on PMA-induced sustained cellular contractile response in rat VSMC in culture. (○) Vehicle and (●)  $10^{-5}$  M ouabain. Values are expressed as mean  $\pm$  SEM ( $n = 6$ ). \* $P < 0.01$  (vs. vehicle).

activation as assessed by  $IP_3$  concentrations. Since either the calcium entry blocker, verapamil, or  $Ca^{2+}$ -free buffer, which completely blocks cellular  $Ca^{2+}$  uptake, abolished the potentiating effect of ouabain on the AVP-induced mobilization of  $[Ca^{2+}]_i$ , the increase in the AVP-mobilized  $[Ca^{2+}]_i$  in the cells preincubated with ouabain appeared to be dependent on enhanced basal and AVP-stimulated cellular  $Ca^{2+}$  uptake by ouabain.

In addition to enhancing  $[Ca^{2+}]_i$ , cellular  $Ca^{2+}$  uptake potentiated by ouabain may also enhance the effect of protein kinase C and thus sustained VSMC contraction. Specifically, the activation of phospholipase C by AVP produces not only  $IP_3$  but also diacylglycerol, a physiological activator of protein kinase C (37), and the activation of protein kinase C is involved in the sustained phase of vascular smooth muscle contraction (39–45). Recent studies in cultured rat VSMC from our laboratory have demonstrated that both PMA and diocanoylglycerol cause the sustained vascular smooth muscle contractile response in the absence of a detectable rise in  $[Ca^{2+}]_i$  (31). Phorbol esters are also known to increase the affinity of protein kinase C for  $[Ca^{2+}]_i$  so that the activation of this enzyme occurs at basal  $[Ca^{2+}]_i$  concentration (46–49). Moreover, the preincubation of VSMC with  $10^{-5}$  M ouabain for 60 min increased basal  $[Ca^{2+}]_i$  concentration to a similar concentration as observed with  $10^{-9}$  M AVP. On this background, the effect of ouabain on the contractile response of VSMC to PMA was investigated as a means to examine whether the effect of inhibition of  $Na^+/K^+$ -ATPase on AVP-induced cellular contraction might involve an enhancement of protein kinase C activity. The results demonstrated that the preincubation of VSMC with  $10^{-5}$  M ouabain for 60 min at  $37^\circ C$  enhanced the sustained VSMC contraction due to submaximal, but not maximal, doses of PMA. This finding is compatible with the observation that a  $Ca^{2+}$  ionophore also enhances the contractile response to PMA (31). These results therefore suggest that the effect of  $Na^+/K^+$ -ATPase inhibition to increase the AVP-induced sustained cellular contractile response may involve an enhancement of protein kinase C activity.

In conclusion, the present results suggest that an increase in cellular  $Ca^{2+}$  uptake induced by the inhibition of  $Na^+/K^+$ -ATPase may enhance the cellular contractile response to AVP by (a) increasing the mobilization of  $[Ca^{2+}]_i$  in the absence of a change in receptor binding or transmembrane signal transduction, and (b) enhancing protein kinase C activation. These findings provide potential insights into the effect of circulating  $Na^+/K^+$ -ATPase inhibitors to enhance the pressor activity of endogenous vasoconstrictors and the effect of calcium channel blockers to protect against cardiac glycoside intoxication (5, 50, 51).

## Acknowledgments

The authors thank Ms. Linda M. Benson for secretarial assistance.

This work was supported by a grant from the National Institutes of Health Program Project DK-19928.

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