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Angiotensin increases Na^+ entry and Na^+/K^+ pump activity in cultures of smooth muscle from rat aorta

(Na^+ transport/Na^+,K^+-ATPase/vascular cell culture/vasoconstriction)

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ABSTRACT **Angiotensin markedly altered the Na^+ permeability of smooth muscle cells cultured from explants of rat aorta. The rate of net Na^+ uptake was followed in the presence of ouabain in order to block Na^+ efflux via the Na^+/K^+ pump. Angiotensin II (AII) or angiotensin III (AIII) increased net Na^+ uptake by about 3-fold. Maximal stimulation of Na^+ uptake was produced by about 10 nM AII. Bradykinin and the angiotensin antagonist [Sar^1, $Ileu^5$, Ala^8]AII had no significant effect on net Na^+ uptake. Angiotensin also enhanced the activity of the Na^+/K^+ pump, which was assayed by following the rate of ouabain-sensitive $^{86}Rb^+$ uptake by the cells. AII and AIII nearly doubled ouabain-sensitive $^{86}Rb^+$ uptake, but bradykinin, norepinephrine, and [Sar^1,$Ileu^5$,Ala^8]AII had no effect. In the presence of ouabain, $^{86}Rb^+$ uptake was not significantly affected by AII or AIII, indicating that angiotensin did not alter passive permeability to Rb^+. Loading the cells with Na^+, either by incubation in K^+-free medium or exposure to the Na^+-selective ionophore monensin, markedly increased ouabain-sensitive $^{86}Rb^+$ uptake. This result indicates that the activity of the Na^+/K^+ pump is limited by the low level of Na^+ that is normally in the cells. AII had no effect on the activity of the Na^+/K^+ pump in Na^+-loaded cells. These results suggest that AII or AIII stimulates the Na^+/K^+ pump in cultured aortic muscle cells by increasing its Na^+ supply.**

The octapeptide hormone angiotensin II (AII) is a highly potent vasoconstrictor (1–3). Specific receptors on the surface of smooth muscle cells (4) mediate the contractile response to AII, which occurs when the level of free Ca^{2+} in the cytoplasm increases (5, 6). It is unclear what transmembrane events are elicited when AII binds to its receptor on the cell surface.

AII is known to depolarize the smooth muscle membrane in various tissues (7–9). Recently, Hamon and Worcel (9) reported evidence suggesting that AII depolarizes uterine smooth muscle by increasing the conductance of the membrane to Na^+. Alterations in Na^+ permeability also appear to be involved in vasoconstriction by angiotensin. Increasing the Na^+ concentration in the medium bathing segments of rat aorta potentiates the contractile response to AII (10), and perfusing rat tail arteries with low Na^+ decreases the constriction elicted by AII (11). However, the reported influences of angiotensin on the Na^+ movements in isolated vascular tissue are ambiguous. Although Friedman and Allardyce (12) reported that angiotensin decreased the Na^+ activity in the medium bathing an artery, Guignard and Friedman (13) found that pressor doses of AII had no such effect.

AII was recently shown to stimulate phosphorylation of the 20,000-dalton light chain of myosin in primary cultures of vascular smooth muscle (14). Therefore, it is feasible to study vasoactive mechanisms in cultured cells. In an attempt to elucidate further the effects of angiotensin on cation transport, we have examined the influence of vasoactive agents on Na^+ and K^+ movements in cultures of rat aortic cells. Cultures of vascular smooth muscle provide a convenient model for investigating the regulation of membrane transport because a homogeneous population of cells is obtained that is free of interference from innervation. In the intact vascular tissue, measurements of cation fluxes and contents are complicated by the presence of cell types other than smooth muscle as well as the binding of cations to sites in the extracellular matrix. We report here that AII increases the Na^+ permeability of aortic muscle cells in culture and the activity of the Na^+/K^+ pump in the pericytoplasmic membrane. Both effects were produced by nearly physiologic levels of AII (1–4). Evidence is presented that the stimulation of the Na^+/K^+ pump by angiotensin results from the enhancement in Na^+ permeability, which would supply the pump with more of its limiting substrate.

MATERIALS AND METHODS

Cell Culture. Primary cultures of vascular smooth muscle were obtained by explanting intimal-medial segments of the thoracic aorta of Sprague–Dawley rats (10–12 weeks old) as described by Ross (15). The explants were grown in 20% fetal bovine serum and medium 199 which was supplemented with extra glucose, amino acids, and vitamins as described by Lewis *et al.* (16).

Stock cultures (75-cm^2 flasks) were passaged by washing once with 2 ml of Ca- and Mg-free Dulbecco's phosphate-buffered saline (P_i/NaCl) and incubating for 5 min at 37°C with 1 ml of 0.05% trypsin in P_i/NaCl containing 0.02% Na_2EDTA. Usually the cultures were passaged twice weekly at a 1:2 split and used for experiments between the 5th and 15th passages. The stock cultures in flasks and in dishes for experiments (Falcon) were grown in medium 199 (GIBCO) containing 10% fetal bovine serum (GIBCO), 10 mM Hepes buffer, 100 units of penicillin G and 100 μg of streptomycin per ml, and 2 μg of butyl *p*-hydroxybenzoate per ml. Culture dishes were seeded at a density of 1×10^5 cells per ml (2 ml for 35-mm dishes and 5 ml for 60-mm dishes). The cells were grown at 37°C in a humidified atmosphere of 10% CO_2/90% air. The culture medium was removed and replaced with serum-free medium 199 when the cultures became confluent, and the cultures were used between 10 and 16 days after plating.

Transport Assays. The rates of $^{86}Rb^+$ uptake (17–19) and net Na^+ uptake (19) were measured as described. The transport

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Abbreviations: AII, angiotensin II; AIII, [des-Asp^1]AII, angiotensin III; P_i/NaCl, Dulbecco's phosphate-buffered saline.

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assay buffer was 120 mM NaCl/5 mM KCl/2 mM $CaCl_2$/1 mM $MgCl_2$/20 mM Hepes adjusted to pH 7.6 with Tris base. Intracellular Na^+ and K^+ were measured as described by Smith and Rozengurt (19). The cultures were washed quickly with 0.1 M $MgCl_2$/10 mM Tris·Hepes to remove extracellular Na^+ and K^+ without diminishing the cellular content of either cation (18, 19). Protein was measured by the method of Lowry *et al.* (20) with bovine serum albumin as a standard. Hormones were obtained from Sigma. Values are means ± SD for 2–10 identically treated cultures.

RESULTS

Angiotensin Enhances Na^+ Entry in Smooth Muscle Cultures. Fig. 1 shows the effect of AII and AIII on the rate of net Na^+ uptake by cultures of vascular smooth muscle derived from rat aorta. Ouabain was present to prevent Na^+ efflux via the Na^+/K^+ pump. During the first 20 min the increase in total cell Na^+ was nearly linear with time. The addition of 0.2 μM AII or AIII increased the rate of net Na^+ uptake by more than 3-fold. Cellular K^+ decreased by an amount that was nearly equal to the amount of Na^+ accumulated. For example, between 10 and 20 min of incubation in the presence of AII and ouabain, K^+ decreased by about 0.024 μmol/mg of protein and Na^+ increased by 0.023 μmol/mg of protein.

AIII appeared to be just as effective as AII in increasing net Na^+ uptake; they produced similar increases in Na^+ uptake at 0.2 μM (Fig. 1) and at 10 nM (Table 1). However, responses to the two angiotensins at shorter periods of exposure might provide a more sensitive comparison of the two peptides. [Sar^1, $Ileu^5$, Ala^8]AII and bradykinin had no significant effect on net Na^+ uptake.

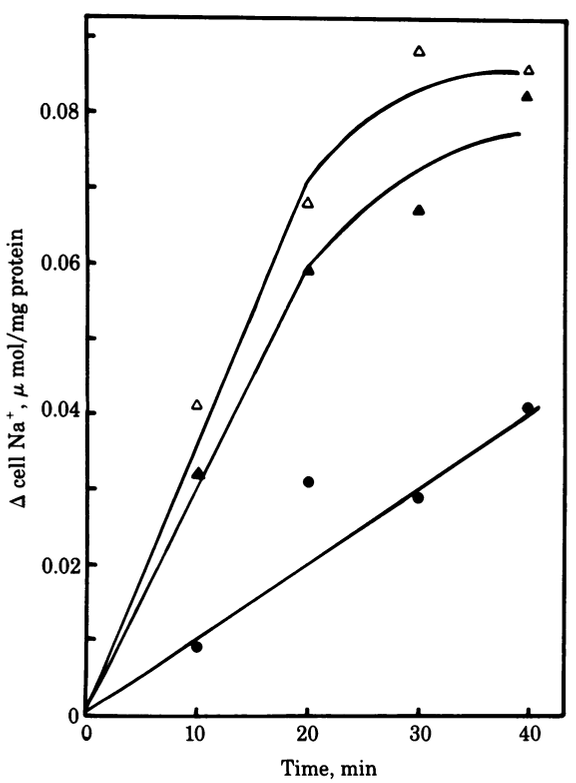

FIG. 1. Effect of 0.2 μM AII (△) or AIII (▲) on the rate of net Na^+ uptake in the presence of ouabain. The rat aortic cultures (60-mm dishes) were washed once with transport assay buffer at 37°C. Then 2 ml of the same buffer containing 2 mM ouabain was added to each dish. The dishes were incubated at 37°C. The amount of Na^+ (0.039 μmol/mg of protein) present in the cells at the start of the experiment was subtracted from the values obtained after incubation. Values are means of duplicate assays. ●, Control (no additions).

Table 1. Effects of vasoactive agents on net Na^+ uptake in the presence of ouabain

Additions	Net Na^+ uptake, μmol/30 min/mg protein	Δ, fold
None	0.044 ± 0.006	1.00
AII	0.111 ± 0.007	2.52
AIII	0.123 ± 0.009	2.80
[Sar^1,Ile^5,Ala^8]AII	0.045 ± 0.001	1.02
Bradykinin	0.048 ± 0.001	1.09

The cultures (60-mm dishes) were washed once with the transport assay buffer and incubated for 30 min at 37°C in 2 ml of the buffer containing 2 mM ouabain and the indicated additions: AII, 10 nM; AIII, 10 nM; [Sar^1,Ile^5,Ala^8]AII, 10 nM; bradykinin, 1 μM. The cultures contained about 0.039 μmol of Na^+ per mg of protein before the experiment. This value was subtracted from each of the values obtained after the incubation. The data presented are means ± SD for two to five identically treated cultures.

A dose–response curve for the effect of AII on net Na^+ uptake is shown in Fig. 2. Maximal stimulation was produced by about 10 nM AII, indicating that near-physiologic levels (1–4) of AII increased the Na^+ permeability of the vascular cell cultures. Moreover, this effect appears to be highly specific for angiotensin because [Sar^1,$Ileu^5$,Ala^8]AII had no significant effect on Na^+ entry (Table 1).

Angiotensin Stimulates the Na^+/K^+ Pump. AII and AIII significantly increased the rate of $^{86}Rb^+$ uptake in the absence of ouabain, but they had little, if any, significant effect on uptake in the presence of ouabain (Fig. 3). These results indicate that angiotensin stimulated only the portion of $^{86}Rb^+$ uptake that is mediated by the Na^+ pump and did not affect passive permeability to Rb^+. In several experiments with smooth muscle cultures from aortic explants of different animals, ouabain-sensitive $^{86}Rb^+$ uptake was increased by 50% to >100% by angio-

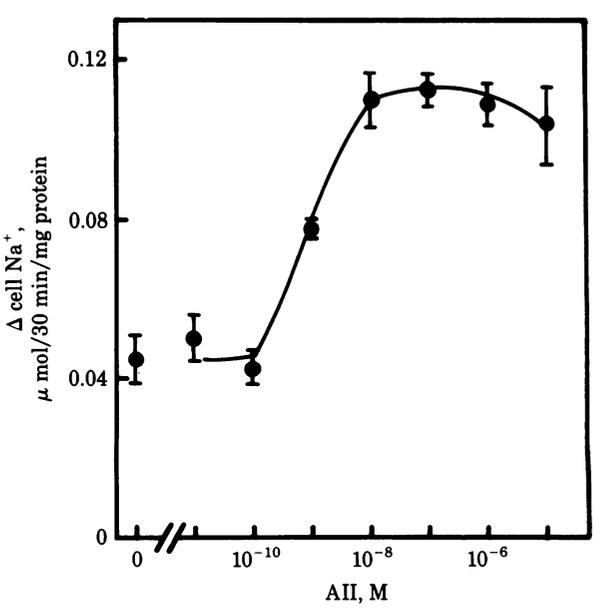

FIG. 2. Effect of AII concentration on net Na^+ uptake in the presence of ouabain. Rat aortic cultures (60-mm dishes) were incubated in the transport assay buffer for 30 min at 37°C with 2 mM ouabain and the indicated concentrations of AII. The amount of Na^+ (0.039 μmol/mg of protein) that was present in the cells at the start of the experiment was subtracted from the values obtained after the incubation. Values are means ± SD for two to five identically treated cultures.

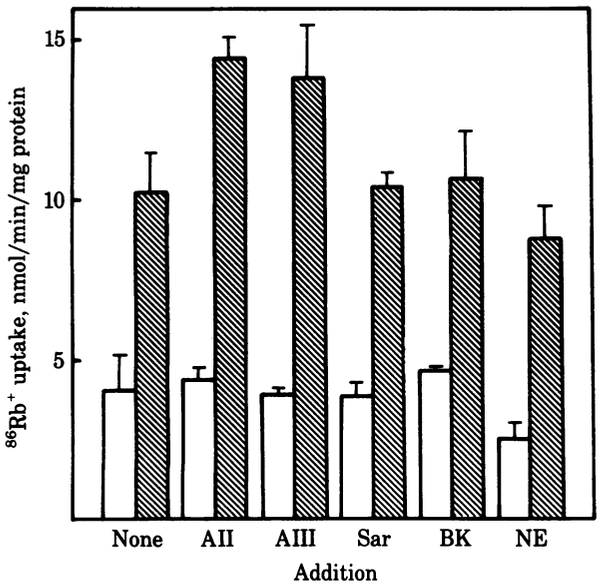

FIG. 3. Effects of vasoactive agents on the rate of $^{86}Rb^+$ uptake by vascular smooth muscle cultures in the presence (open bars) and absence (hatched bars) of 2 mM ouabain. The rate of $^{86}Rb^+$ uptake was assayed after a 20-min incubation at 37°C with the indicated additions: AII, 20 nM; AIII, 20 nM; [Sar^1,Ileu^5,Ala^8]AII, 20 nm (Sar); bradykinin, 2 μM (BK); norepinephrine, 2 μm (NE). Values are means ± SD for two to six identically treated cultures.

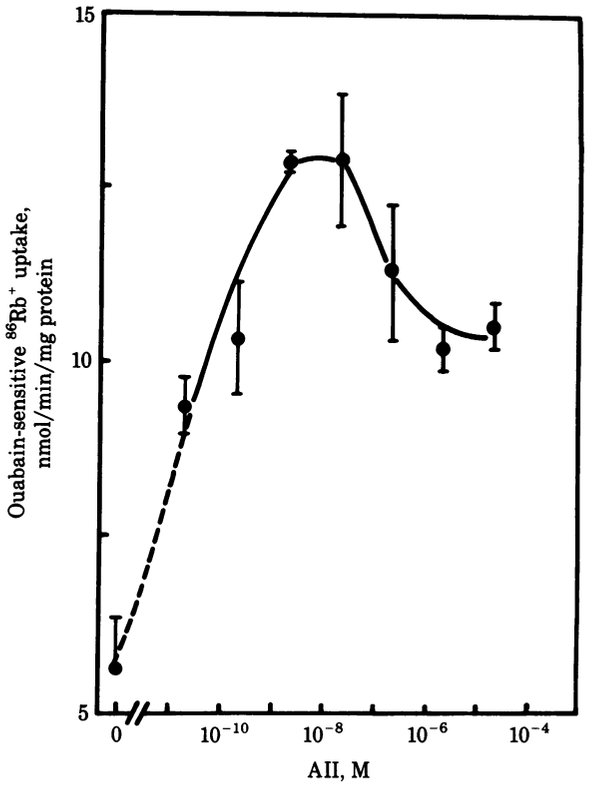

FIG. 4. Effect of AII concentration on the rate of ouabain-sensitive $^{86}Rb^+$ uptake. Rat aortic cell cultures (35-mm dishes) were incubated in 1 ml of the transport assay buffer at 37°C for 20 min in the presence or absence of 2 mM ouabain. Then $^{86}RbCl$ was added and the incubation was continued for 10 min at 37°C. AII had no significant effect on $^{86}Rb^+$ uptake in the presence of ouabain. This rate (4.9 ± 0.6 nmol/min per mg of protein) was subtracted from the total uptake values to obtain the ouabain-sensitive portion.

tensin. Neither [Sar^1,Ileu^5,Ala^8]AII nor bradykinin had any significant effect on $^{86}Rb^+$ uptake in the presence or absence of ouabain. Norepinephrine selectively decreased the rate of ouabain-insensitive $^{86}Rb^+$ uptake. Fig. 4 shows a dose–response curve for the effect of AII on the rate of ouabain-sensitive $^{86}Rb^+$ uptake. Maximal stimulation was 2-fold and occurred at approximately 5 nM AII. These results indicate that physiological levels of AII and AIII enhance Na^+/K^+ pump activity in the aortic muscle cultures.

Na^+ Loading Increases $^{86}Rb^+$ Uptake. It appeared that the stimulation of the Na^+ pump by angiotensin might result from the greater permeability to Na^+ of cultures treated with AII. Normally, the Na^+ pump is limited in activity by the low level of Na^+ in animal cells. For example, in Swiss 3T3 cells, the Na^+ ionophore monensin increases the rate of ouabain-sensitive $^{86}Rb^+$ uptake by about 8-fold by overcoming this substrate limitation (19). Monensin increased ouabain-sensitive $^{86}Rb^+$ uptake by about 4-fold in the aortic cultures (Fig. 5). Loading the cells with Na^+ by incubation in a K^+-free buffer increased ouabain-sensitive $^{86}Rb^+$ uptake by the same degree as did monensin. Monensin increased cell Na^+ from 0.032 μmol/mg of protein to 0.23 μmol/mg of protein and the K^+-free incubation increased cell Na^+ to 0.28 μmol/mg of protein.

Angiotensin Does Not Increase $^{86}Rb^+$ Uptake in Na^+-Loaded Cells. If angiotensin increases Na^+ pump activity by supplying it with more of its limiting substrate, Na^+, then AII would not be expected to increase pump activity in cells that have been loaded with Na^+. Fig. 5 shows that AII had no significant effect on ouabain-sensitive $^{86}Rb^+$ uptake by the Na^+-loaded cells. Treatment with cycloheximide at 30 μg/ml did not diminish the stimulatory effect of AII on ouabain-sensitive $^{86}Rb^+$ uptake. These results support the hypothesis that AII increases the activity of preexisting Na^+/K^+ pumps in aortic muscle cultures by increasing the availability of Na^+ to the pump.

We have observed that monensin stimulates ouabain-sensitive $^{86}Rb^+$ uptake under a wide variety of conditions. However, in Na^+-loaded cells, monensin inhibited the pump (Fig. 5), even though total cell Na^+ was increased further by the monensin treatment (0.37 μmol/mg of protein). The inhibition of the pump may be related to the severe depletion of cell K^+ by monensin in the already Na^+-loaded cells.

Finally, the stimulatory effects of AII on net Na^+ uptake in the presence of ouabain and Na^+ pump activity have been observed in cultures of aortic media from five different rats as well as in cultures that have been cloned and passaged for several months. Therefore, these modulatory effects of angiotensin on Na^+ and K^+ transport appear to be a general and stable feature of smooth muscle cultured from rat aorta.

DISCUSSION

The results presented here suggest that angiotensin activates a Na^+ channel in vascular smooth muscle. The rate of net Na^+ uptake by the aortic muscle cultures was greatly enhanced by AII (Figs. 1 and 2). Angiotensin appears to increase Na^+ permeability selectively because the passive uptake of $^{86}Rb^+$ in the presence of ouabain was not significantly affected by AII or AIII (Fig. 3). Specific receptors on the cell surface probably mediate the increase in Na^+ permeability because it occurred at AII concentrations of 1 nM or less. Moreover, [des-Asp^1]AII appeared to be just as potent as AII, but [Sar^1,Ileu^5,Ala^8]AII was inactive. An aromatic amino acid in position 8 is known to be critical for contractile and other agonist activities of angiotensin, whereas removing the NH_2-terminal aspartic acid from AII produces AIII which is a highly active vasoconstrictor (3). Moreover, [Sar^1,Ileu^5,Ala^8]AII prevented AII from increasing either

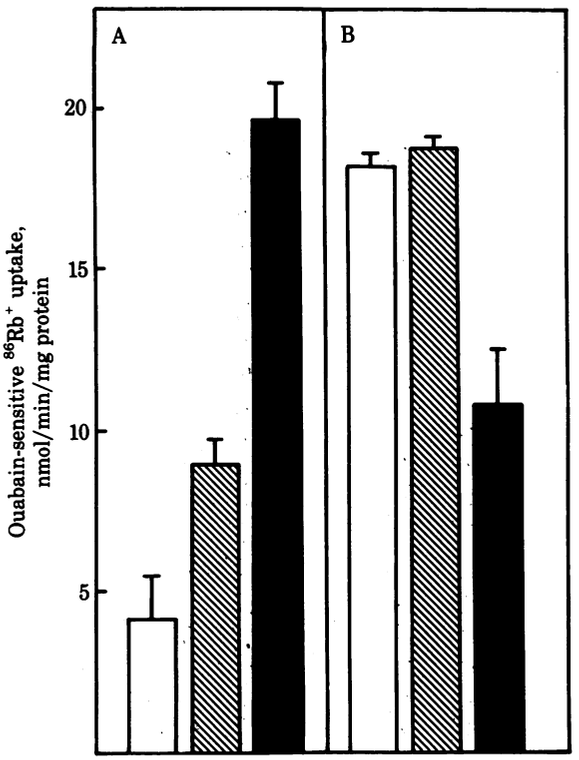

FIG. 5. Effect of AII on ouabain-sensitive $^{86}Rb^+$ uptake by cultured aortic cells that had been loaded with Na^+ by incubation for 3 hr at 37°C in 2 ml of buffer (120 mM NaCl/2 mM $CaCl_2$/1 mM $MgCl_2$/10 mM glucose/20 mM Hepes, pH 7.6). The control cells were incubated in the same buffer plus 5 mM KCl. During the last 20 min of the 3-hr period, AII was added to a final concentration of 0.2 μM. The medium was removed and 1 ml of transport assay buffer was added plus AII, 0.2 μM (hatched bars) or monensin, 20 μg/ml (solid bars). Open bars, no additions. After a 5-min incubation at 37°C, $^{86}Rb^+$ was added for 10 min. The Na^+-loaded cells (*B*) contained about 0.285 μmol/mg of protein compared to 0.032 for the control cells (*A*). Uptake in the presence of 2 mM ouabain (4.1 ± 0.6 for Na^+-loaded cells and 4.6 ± 0.8 for control cells) was subtracted from total uptake to obtain the ouabain-sensitive portion.

net Na^+ uptake or $^{86}Rb^+$ uptake in myomedial cultures obtained from rat aorta by enzymatic dispersion (21).

Bradykinin had no significant effect on either net Na^+ uptake (Table 1) or $^{86}Rb^+$ uptake (Fig. 3). Alexander and Gimbrone (22) reported that both bradykinin and angiotensin stimulate prostaglandin E synthesis in muscle cultures from human umbilical vein. If prostaglandin synthesis is also stimulated by bradykinin and angiotensin in muscle cultures from rat aorta, then the increases in Na^+ and K^+ transport that we observed are probably not due to an effect on prostaglandin synthesis because they were produced by angiotensin but not bradykinin.

Although increasing the permeability of a cell to Na^+ might depolarize its membrane, we have not studied the effect of AII on membrane potential in our aortic muscle cultures. Recently, Zelcer and Sperelakis (23) reported that AII depolarizes reaggregates of smooth muscle cells prepared from rat aorta. Replacing all of the Na^+ in the solution bathing the aggregates with Tris or choline resulted in a loss of the response to AII. Previously it was shown (9) that the depolarization of uterine smooth muscle was dependent on the presence of external Na^+. In the rat uterus preparation, the contractile response to AII showed a phasic component which may have been triggered by Ca^{2+} influx and a tonic phase which the authors suggested was due to the release of Ca^{2+} sequestered within the cell (8). The higher permeability of the cell to Na^+ might increase free Ca^{2+} via a Na^+/Ca^{2+} exchange as discussed by Blaustein and others (24–28). Alternatively, depolarization might activate a Ca^{2+} transporter that is regulated by the membrane potential (28). We recognize that the available evidence does not rule out the possibility that AII directly alters Ca^{2+} transport and secondarily produces the changes in Na^+ translocation that we observed. Angiotensin alters $^{45}Ca^{2+}$ fluxes and stimulates aldosterone synthesis in glomerulosa cells isolated from bovine adrenals (29), but it is not known how AII produces these effects.

The depolarization that AII produces in rat uterus and aortic muscle reaggregates is transient. It lasts for only 6–8 sec in the smooth muscle reaggreates (23). In contrast, the increase in Na^+ permeability that we observed (Fig. 1) lasted for 30 min or longer in the continued presence of AII. However, AII also stimulated the Na^+/K^+ pump (Figs. 3 and 5), which is electrogenic (30). Faster Na^+ pumping in the presence of AII might repolarize the membrane even while Na^+ permeability remains high.

Additionally, a defective tachyphylactic mechanism may be responsible for the prolonged nature of the effect of angiotensin on Na^+ and K^+ movements in vascular cultures. The contractile response of intact aortic tissue from rats rapidly develops tachyphylaxis to AII (31). On the other hand, it is not known if a continuous exposure of smooth muscle to AII *in vivo* produces a prolonged increase in Na^+ permeability. Villamil *et al.* (32) observed that a continuous infusion of AII in dogs for 5–6 weeks increased the Na^+ content of the carotid arterial wall. We recently observed that exposing aortic muscle cultures to AII for a period of 4 hr produced a 50% decrease in the apparent number of AII receptors (21). This so-called receptor down-regulation that we have observed in cultures of vascular cells has been suggested to be involved in tachyphylaxis to AII *in vivo* (33). However, the rate of receptor down-regulation may be much slower in cell cultures than it is *in vivo*.

The following observations suggest that AII activates the pump in cultures of vascular smooth muscle by increasing its Na^+ supply: (*i*) increasing cell Na^+ strongly activated the Na^+ pump, indicating that Na^+ is a limiting substrate for the pump in the smooth muscle cultures (Fig. 5) as we previously reported in Swiss 3T3 cells (19); (*ii*) AII has no effect on Na^+ pump activity in Na^+-loaded cells (Fig. 5) or in the presence of the Na^+ ionophore monensin (21); (*iii*) AII increases the Na^+ permeability of the muscle cultures (Figs. 1 and 2); (*iv*) the AII antagonist [Sar^1,Ile^5,Ala^8]AII increased neither Na^+ permeability nor Na^+ pump activity in the cultures (Table 1; Fig. 3). Furthermore, we recently found that AII had no effect on pump activity in the absence of extracellular Na^+ (unpublished data).

AII did not appear to increase the total Na^+ content of the smooth muscle cultures unless Na^+ efflux via the Na^+/K^+ pump was blocked by ouabain (data not shown). Probably not all of the Na^+ in a cell—for example, that in the nucleus—is immediately available to the pump on the cell surface. Clearly, fluctuations in membrane permeability to Na^+ may modulate the level of Na^+ in a pool just beneath the cell surface without producing detectable changes in total Na^+ content. In fact, our studies (19) with the Na^+ ionophore monensin suggested that the activity of the Na^+/K^+ pump is a sensitive indicator of Na^+ activity in the vicinity of the cytoplasmic surface of the cell membrane. The observation that AII increased Na^+ pump activity only under conditions such that cell Na^+ is a limiting substrate for the pump suggests that AII does in fact increase intracellular Na^+ in the absence of ouabain, at least in the vicinity of the plasma membrane.

Interestingly, Turker *et al.* (34) observed that AII increases $^{22}Na^+$ efflux from rat uterine smooth muscle. Although this effect was blocked by ouabain at 2.5 ng/ml, this level of ouabain

is probably too low to inhibit the Na^+/K^+ pump in rat tissues (35). Angiotensin peptides are potent stimulators of intestinal Na and water absorption; however, this effect of AII was shown to be due to the release of norepinephrine from nerve endings in the jejunum (36). The Na^+/K^+-ATPase activity of microsomal membranes prepared from certain tissues, but not others, was increased when assayed in the presence of AII (37–39). Although membranes from vascular smooth muscle were not included in previous studies (37–39), we have found that AII had no significant effect on the Na^+/K^+-ATPase activity in membranes isolated from the smooth muscle cultures (unpublished data). The lack of an allosteric effect of AII on ATPase activity is consistent with the absence of an effect of AII on $^{86}Rb^+$ uptake in Na^+-loaded cells (Fig. 5).

Even after many generations in culture, the muscle cells from rat aortic explants were able to modulate membrane Na^+ and K^+ transport in response to a vasoactive agent that acts through specific receptors on the cell surface. Thick myosin filaments appear to be absent from some vascular smooth muscle cultured from explants (40). Because we have been unable to detect thick filaments by electron microscopy of our smooth muscle cultures (unpublished data), it would appear that the contractile elements are elaborated independently from the excitatory systems in the sarcolemmal membrane.

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