Angiotensin II rapidly increases phosphatidate-phosphoinositide synthesis and phosphoinositide hydrolysis and mobilizes intracellular calcium in cultured arterial muscle cells

(smooth muscle/inositol triphosphate/aorta/calcium transport)

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ABSTRACT Smooth muscle cells were cultured from rat thoracic aorta and labeled to a stable specific activity with $^{45}\text{Ca}^{2+}$, *myo*-[2- ^3H]inositol, or ^{32}P . The efflux of $^{45}\text{Ca}^{2+}$ was monitored over 10-sec intervals. Angiotensin II (AII) increased the amount of $^{45}\text{Ca}^{2+}$ lost by 5-fold in the first 10-sec interval after the addition of AII and by 10-fold in the second 10-sec interval. AII-stimulated $^{45}\text{Ca}^{2+}$ release was blocked by the angiotensin antagonist [1-sarcosine, 8-leucine]AII and by La^{3+} . The removal of external Ca^{2+} had no effect on AII-stimulated $^{45}\text{Ca}^{2+}$ release. Depolarization with high external K^+ only slightly increased $^{45}\text{Ca}^{2+}$ efflux and had no effect on AII-induced $^{45}\text{Ca}^{2+}$ release. AII had no effect on the initial rate of $^{45}\text{Ca}^{2+}$ influx. These results indicate that the rapid $^{45}\text{Ca}^{2+}$ efflux evoked by AII is probably due to the release of $^{45}\text{Ca}^{2+}$ sequestered intracellularly rather than to an increase in the Ca^{2+} permeability of the plasma membrane. AII provoked rapid increases in the levels of phosphatidic acid and phosphoinositides in the cells. These increases in phospholipids were associated with increases in phospholipase C-generated inositol phosphates (tri-, di-, and mono-). It appears that AII simultaneously increases phosphoinositide hydrolysis and synthesis in vascular smooth muscle, and both phospholipid effects may contribute to inositol triphosphate generation, which was sufficiently rapid to have a role in intracellular Ca^{2+} mobilization.

Angiotensin II (AII) is a potent vasoconstrictor that acts directly on vascular smooth muscle (1, 2). The binding of the hormone to a receptor on the surface of the smooth muscle cell (SMC) (3-6) somehow increases cytoplasmic calcium activity, which evokes a contractile response. The initial phase of the contractile response to AII (7) and other agonists (7-11) appears to be dependent on intra- rather than extracellular Ca^{2+} . In contrast, the slow tonic component of agonist-induced contraction depends on extracellular Ca^{2+} (7-11).

SMC cultured from rat aorta respond to AII by increasing the cycling of Na^+ in and out of the cell (12-14). AII stimulates Na^+ entry via an amiloride-inhibitable transporter, which results in an increased supply of Na^+ to the Na^+/K^+ pump. Na^+/K^+ pump activity is thereby increased because cellular Na^+ is the rate-limiting substrate for the pump in mammalian cells including vascular smooth muscle *in vivo* and in culture (15-17). AII depolarizes cultured aortic SMC by 10-15 mV as indicated by reciprocal changes in the steady-state distributions of [^3H]tetraphenylphosphonium $^+$ and $^{35}\text{SCN}^-$ (18).

Recent evidence has suggested that inositol 1,4,5-(PO_4) $_3$

(Ins- P_3), which is generated by agonist-induced hydrolysis of triphosphoinositide (19, 20), releases calcium from a nonmitochondrial site, presumably the endoplasmic reticulum (21-23). AII has been shown to provoke TPI breakdown in rat liver (24) and adrenal glomerulosa cells (25), and the effect of AII in the latter tissue is apparently associated with an increase in *de novo* synthesis of phosphoinositides (26). In the present studies, we have examined the effects of AII on phospholipid metabolism, inositol phosphate generation, and calcium mobilization in SMC cultured from rat aorta.

MATERIALS AND METHODS

Cell Culture. Primary cultures were initiated from cell suspensions obtained by incubating thoracic aortae from 12 Sprague-Dawley rats with collagenase and elastase as described (13). The tunica adventitia was removed as an everted tube after a short incubation. The remaining tubes of tunica media were minced and incubated with the enzymes to obtain $\approx 2 \times 10^6$ viable SMC per aorta as determined by trypan blue exclusion and counting in a hemocytometer. The SMC were plated at $1 \times 10^5/\text{ml}$ in 2 ml of medium 199 containing 10% (vol/vol) fetal bovine serum (no antibiotics) in 35-mm-diameter culture dishes (Falcon). Five days later the quiescent, nongrowing cultures, derived from stock cultures that had been passaged (1:3) 5-15 times, were incubated with the radioisotopes. Cultures seeded at the same time contained a highly uniform amount of total protein as measured by the method of Lowry *et al.* (27).

Calcium Transport. The culture medium was removed by aspiration, and the SMC were rinsed twice at 37°C with transport buffer (120 mM NaCl/5 mM KCl/1 mM $\text{MgCl}_2/0.5$ mM $\text{CaCl}_2/10$ mM glucose/20 mM Hepes adjusted to pH 7.4 with Tris base). One milliliter of this buffer and 10 μCi of $^{45}\text{Ca}^{2+}$ (1 Ci = 37 GBq) were added and the cultures were incubated at 37°C for 3 hr. Then the cultures were rapidly rinsed eight times (20 sec total) with transport buffer and 1 ml of the buffer was added. The medium was removed and replaced with 1 ml of transport buffer at 1 and 2 min and thereafter at 10-sec intervals. AII and other compounds were added as indicated during the time course of $^{45}\text{Ca}^{2+}$ efflux. The amount of $^{45}\text{Ca}^{2+}$ lost from the cells in each time interval was measured by liquid scintillation counting with 4.5 ml of Hydrocount (Baker). Representative experiments, which were repeated at least three times, are shown in Figs. 1-3.

$^{45}\text{Ca}^{2+}$ uptake was assayed at 37°C after incubating cultures in transport buffer as described above. Then the cultures were incubated for 10, 20, 30, or 60 sec with 2 μCi of

Abbreviations: AII, angiotensin II; [Sar 1 , Leu 8]AII, [1-sarcosine, 8-leucine]AII; SMC, smooth muscle cell(s); PtdIns, phosphatidylinositol; PtdOH, phosphatidic acid; Ins- P_3 , inositol 1,4,5-(PO_4) $_3$; Ins- P_2 , inositol 1,4-(PO_4) $_2$; Ins- P , inositol 1-phosphate.

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$^{45}\text{Ca}^{2+}$ in the presence or absence of $0.2\ \mu\text{M}$ AII in 1 ml of transport buffer. To terminate uptake, external $^{45}\text{Ca}^{2+}$ was removed by rinsing the culture eight times at 2°C with $0.1\ \text{M}$ MgCl_2 containing $10\ \text{mM}$ Hepes and $10\ \text{mM}$ LaCl_3 to inhibit $^{45}\text{Ca}^{2+}$ efflux. Intracellular radioactivity was extracted with $0.1\ \text{M}$ HNO_3 and measured by liquid scintillation counting. Zero time blanks, which were done by rinsing cultures twice with the La/Mg solution before adding the $^{45}\text{Ca}^{2+}$ uptake solution and immediately stopping the uptake as described above, were $\approx 300\ \text{cpm}$ per culture (equal to about $1/10$ th the total uptake after 20 sec at 37°C).

Phospholipid Metabolism. $^{32}\text{P}_i$ ($20\ \mu\text{Ci}$) or $2\ \mu\text{Ci}$ of *myo*-[2- ^3H]inositol was added to the culture medium 4 to 5 days after seeding. The isotopes were present long enough to label phospholipids to constant specific activity. In most cases, the isotopes were present for 48–72 hr but, on a few occasions, $^{32}\text{P}_i$ was present for only 3 hr to label TPI and diphosphoinositide to constant specific activity because these phospholipids turn over much more rapidly than other phospholipids. After the cultures were labeled to constant specific activity, AII was added to medium, and they were returned to the CO_2 incubator for specified time periods. For phospholipid analysis, 1 ml of ice-cold methanol was added to each culture after aspirating the medium to terminate the incubation. The cells were scraped from the dish, and phospholipids were extracted with acidified chloroform/methanol, identified, and quantified as described (25, 26).

In the experiments in which [^3H]inositol phosphates were examined, $10\ \text{mmol}$ of LiCl_2 was added to the culture medium 15 min prior to the addition of AII to inhibit inositol-phosphatase(s) and allow accumulation of inositol phosphates. Incubations were stopped by aspirating the medium and adding 1 ml of ice-cold 5% trichloroacetic acid. [^3H]inositol phosphates were separated (after ether extraction to remove trichloroacetic acid) by Dowex-1 formate chromatography as described by Berridge *et al.* (28).

Materials. $^{45}\text{CaCl}_2$ ($2.1\ \text{mCi}/135\ \mu\text{g}$) was obtained from Amersham, *myo*-[2- ^3H]inositol ($15.8\ \text{Ci}/\text{mmol}$) and carrier-free $^{32}\text{P}_i$ were from New England Nuclear. Medium 199 and trypsin (2.5% porcine pancreas, papovavirus tested) used for subculturing the cells were from GIBCO. Fetal bovine serum was from Hyclone (Logan, UT), AII (human, synthetic form, acetate salt) was from Sigma, and [1-sarcosine, 8-leucine]AII ([Sar 1 , Leu 8]AII) was from Peninsula Laboratories (San Carlos, CA).

RESULTS

Angiotensin Stimulates $^{45}\text{Ca}^{2+}$ Efflux. The effect of AII on $^{45}\text{Ca}^{2+}$ efflux from cultured SMC is shown in Fig. 1. In the absence of AII, a nearly constant amount of $^{45}\text{Ca}^{2+}$ was released from the cells in each 10-sec interval. In the first 10-sec interval after addition of AII, the amount of $^{45}\text{Ca}^{2+}$ appearing in the external medium increased by 5-fold, and the amount of $^{45}\text{Ca}^{2+}$ released into the medium in the second 10-sec interval was ≈ 10 times that in the absence of AII. Thereafter, the amount of $^{45}\text{Ca}^{2+}$ released per 10-sec interval decreased, eventually to almost the control level.

The optimal concentration of AII for evoking the rapid release of $^{45}\text{Ca}^{2+}$ was $\approx 100\ \text{nM}$ with higher or lower concentrations causing less than optimal $^{45}\text{Ca}^{2+}$ release (Fig. 1). The AII antagonist [Sar 1 , Leu 8]AII (29) failed to increase $^{45}\text{Ca}^{2+}$ release (Fig. 2). The addition of $2\ \mu\text{M}$ [Sar 1 , Leu 8]AII at the same time as $20\ \text{nM}$ AII completely prevented AII from stimulating $^{45}\text{Ca}^{2+}$ release. These results indicate that $^{45}\text{Ca}^{2+}$ release is mediated by an angiotensin receptor.

The time course of $^{45}\text{Ca}^{2+}$ uptake by the cells approximated an exponential approach to equilibrium. Mean $^{45}\text{Ca}^{2+}$ uptake values in the presence of $0.2\ \mu\text{M}$ AII did not differ by more than 5% from control values at each time point (three experiments done in duplicate). These data indicate that

FIG. 1. Effect of AII concentration on $^{45}\text{Ca}^{2+}$ efflux from cultured aortic muscle cells. AII: $\circ$, none; $\bullet$, $20\ \text{pM}$; Δ , $200\ \text{pM}$; $\blacktriangle$, $2\ \text{nM}$; $\square$, $20\ \text{nM}$; $\blacksquare$, $200\ \text{nM}$; ∇ , $2\ \mu\text{M}$. AII was added at 40 sec (arrow).

there is no detectable change in Ca^{2+} permeability of the plasma membrane over time interval and conditions of AII treatment that greatly increase $^{45}\text{Ca}^{2+}$ efflux.

Effects of La^{3+} , High K^+ , and EGTA on AII-Evoked $^{45}\text{Ca}^{2+}$ Release. Addition of $1\ \text{mM}$ La^{3+} moderately inhibited $^{45}\text{Ca}^{2+}$ efflux in the absence of AII (data not shown). However, $1\ \text{mM}$ La^{3+} almost completely blocked the stimulatory effect of AII (Fig. 3). This finding indicates that AII releases intracellular $^{45}\text{Ca}^{2+}$ rather than $^{45}\text{Ca}^{2+}$ bound extracellularly. Under these conditions, there appears to be little or no $^{45}\text{Ca}^{2+}$ bound extracellularly since La^{3+} is known to displace external calcium, which would increase, rather than inhibit, $^{45}\text{Ca}^{2+}$ release (30).

Addition of $70\ \text{mM}$ K^+ (substituted for Na^+) only slightly increased rapid $^{45}\text{Ca}^{2+}$ release (Fig. 3). A slower component of $^{45}\text{Ca}^{2+}$ release was markedly increased both by AII and higher external K^+ (results to be published elsewhere). Experiments with [^3H]tetraphenylphosphonium $^+$ indicate that $70\ \text{mM}$ K^+ decreases the membrane potential from about $-60\ \text{mV}$ to almost 0 (18). AII induced slightly less $^{45}\text{Ca}^{2+}$

FIG. 2. The AII antagonist [Sar 1 , Leu 8]AII prevents AII from rapidly releasing $^{45}\text{Ca}^{2+}$. AII and [Sar 1 , Leu 8]AII were used at $20\ \text{nM}$ and $2\ \mu\text{M}$, respectively. Additions: $\circ$, none; $\bullet$, AII; Δ , [Sar 1 , Leu 8]AII; $\blacktriangle$, AII plus [Sar 1 , Leu 8]AII. Additions were made at 60 sec (arrow).

FIG. 3. Effects of lanthanum, high K^+ , and EGTA on $^{45}Ca^{2+}$ efflux in the presence and absence of AII. AII, $LaCl_3$, and EGTA were used at 20 nM, 1 mM, and 0.1 mM, respectively. Medium containing EGTA and no added Ca^{2+} was used to rinse the cultures eight times at the start of and throughout the efflux period to test the effect of AII in the absence of external Ca^{2+} . Additions: $\circ$, none; $\bullet$, AII; ∇ , 70 mM K^+ /AII; Δ , EGTA/AII; $\blacksquare$, La^{3+} /AII; ∇ , 70 mM K^+ . Additions were made at 40 sec (arrow).

release in the presence of 70 mM K^+ as compared with the usual 5 mM K^+ (Fig. 3). Deletion of calcium from the transport buffer [with or without (data not shown) addition of 0.1 mM EGTA] had no effect on AII stimulation of $^{45}Ca^{2+}$ efflux (Fig. 3).

Angiotensin Increases Phosphatidate-Phosphoinositide Synthesis. Increases in phosphatidic acid (PtdOH) were evident within 30 sec of addition of AII to ^{32}P -prelabeled cultured SMC (Fig. 4). (Since changes in phospholipids were studied after constant specific activity had been reached, ^{32}P content will reflect changes in phospholipid mass rather than changes in turnover.) The increase in PtdOH could occur as a result of either increases in *de novo* synthesis of PtdOH or hydrolysis of phosphoinositides. To gain further insight into these possibilities, AII-induced changes in the levels of phosphoinositides were examined (Fig. 5). As shown, although changes in TPI levels were not statistically significant, there appeared to be a small decrease during the first

FIG. 4. Effect of AII on PtdOH in vascular SMC. Cell cultures were labeled for 48 hr with $^{32}PO_4$ and then treated with 100 nM AII for the indicated times. Values are mean $\pm$ SEM of four plates.

* $P < 0.05$ (vs. control) by a standard *t* test.

FIG. 5. Effect of AII on triphosphoinositide (A) and diphosphoinositide (B) in vascular SMC. Cell cultures were incubated with $^{32}PO_4$ for 48 or 3 hr to label polyphosphoinositides to constant specific activity. AII (100 nM) was then added; and incubation was continued. Values are mean $\pm$ SEM of eight determinations. Results have been normalized (i.e., each value is expressed as percentage of mean control for each experiment to minimize differences in total $^{32}PO_4$ incorporation between experiments). *P* (vs. control) was determined by standard *t* test: *, <0.005 ; †, 0.025; ‡, 0.005.

10 sec, followed by a slight increase to levels 15–20% above the control level after 1 min of AII treatment. Diphosphoinositide, on the other hand, simply showed progressive increases, which were statistically significant within 1 min of AII stimulation. Peak increases of 50–100% for diphosphoinositide were observed after 2–5 min of AII treatment.

Dose-related effects of AII on phosphatidylinositol (PtdIns), diphosphoinositide, and PtdOH are shown in Fig. 6. Nearly two-fold increases in PtdOH and diphosphoinositide were observed at 100 nM AII. Twenty-five-percent increases in PtdIns were also observed at 100 nM AII and, although this was not statistically significant in this set of observations, similar increases (17–35%) were observed repeatedly in other experiments, and pooled results observed at 10 and 100 nM AII were statistically significant [percent increase (mean $\pm$ SEM) = 22 ± 2 ($n = 10$); $P < 0.001$]. Note that, in the dose-response curves shown in Fig. 6, changes in diphosphoinositide and PtdIns were parallel and occurred at relatively lower agonist concentrations than those that increase PtdOH. This may reflect the fact that PtdOH is derived from both *de novo* synthesis (from glycerol-3'- PO_4 and fatty acyl-CoA) and hydrolysis of phosphoinositides. Increases in PtdOH do not appear to mediate AII-evoked Ca^{2+} release since much higher concentrations of AII were needed to increase PtdOH than to release intracellular $^{45}Ca^{2+}$.

Angiotensin Increases Phosphoinositide Hydrolysis. Although triphosphoinositide levels did not change significantly, phospholipase C-mediated turnover of triphosphoinositide was nevertheless evident. As shown in Fig. 7, AII increased the generation of Ins- P_3 by $\approx 50\%$ within 20 sec of treatment. Ins- P_3 generation after 10 and 20 sec of AII treatment was statistically significant (Table 1). Furthermore, these increases in Ins- P_3 could not be explained by diminished conversion of Ins- P_3 to inositol 1,4-diphosphate (Ins-

FIG. 6. Dose-related effects of AII on phospholipid levels in vascular SMC. (A) PtdOH. (B) Diphosphoinositide. (C) PtdIns. Cell cultures were labeled for 48 hr with $^{32}\text{PO}_4$ and then treated for 5 min with AII. Values are mean $\pm$ SEM of four determinations. P (vs. control) was determined by standard t test: *, <0.05 ; †, <0.025 ; ‡, <0.01 ; §, <0.005 ; ¶, <0.001 .

P_2) and inositol 1-phosphate (Ins- P), as the latter two substances were also significantly increased (by 37% and 28%, respectively; Fig. 7 and Table 1).

The effect of AII on inositol phosphate production was also tested under the conditions used to assay $^{45}\text{Ca}^{2+}$ efflux. Cultures (100 mm) were labeled with 5 μCi of [^3H]inositol for

FIG. 7. Effect of AII on generation of Ins- P_3 (A), Ins- P_2 (B), and Ins- P (C) in vascular SMC. Cell cultures were labeled for 72 hr with [^3H]inositol and then treated with 100 nM AII for the indicated times. Values are mean $\pm$ SEM of four determinations.

Table 1. Effects of AII on inositol phosphate levels in cultured aortic SMC

Substance	[^3H]Inositol phosphate, cpm per culture		
	Control	AII (100 nM)	P
Ins- P_3	287 $\pm$ 31	418 $\pm$ 50	<0.05
Ins- P_2	339 $\pm$ 29	466 $\pm$ 38	<0.025
Ins- P	1296 $\pm$ 39	1523 $\pm$ 65	<0.01

Cultures were labeled with 2 μCi of [^3H]inositol for 72 hr. Then, AII was added to the culture medium as indicated. After 10 or 20 sec, incubation was stopped by aspirating the medium and adding trichloroacetic acid. Since there were no significant differences between the 10- and 20-sec data (Fig. 7), they were combined. Values are mean $\pm$ SEM for eight determinations. P (angiotensin treatment vs. control) was determined by standard t test.

4 days (from the day after subculturing until the day of the experiment). Then the cultures were rinsed and incubated in efflux buffer for 1 hr. Li^+ was not used in this experiment. AII (0.2 μM) was added for 30 sec to the experimental, but not to the control, cultures. The medium was removed, and inositol phosphates were extracted with 0.1 M formic acid and separated chromatographically (28). AII increased Ins- P from 3221 $\pm$ 227 to 6718 $\pm$ 106, Ins- P_2 from 792 $\pm$ 62 to 4700 $\pm$ 298, and Ins- P_3 from 1281 $\pm$ 162 to 1990 $\pm$ 190 [values are cpm per culture (mean $\pm$ SEM); $n = 4$]. These results show that AII rapidly increases phosphoinositide hydrolysis and mobilizes intracellular calcium under essentially identical conditions.

DISCUSSION

The present results indicate that AII rapidly releases sequestered $^{45}\text{Ca}^{2+}$ from cultured aortic SMC. Previously, Deth and van Breeman (11) reached this conclusion from studies of the effect AII on contraction and $^{45}\text{Ca}^{2+}$ efflux from intact vascular tissue. The fact that membrane depolarization with high external K^+ failed to cause rapid $^{45}\text{Ca}^{2+}$ efflux from cultured SMC indicates that membrane depolarization is insufficient to evoke rapid $^{45}\text{Ca}^{2+}$ release. Similarly, the contractile effect of AII on vascular smooth muscle is independent of changes in membrane potential since AII evokes contraction in the presence of depolarizing concentrations of K^+ (2). The effect of AII on $^{45}\text{Ca}^{2+}$ efflux is also independent of cAMP since AII has no significant effect on cellular cAMP in cultured aortic SMC (13). Moreover, increasing the cAMP content of the cells by β -adrenergic stimulation with isoproterenol inhibits $^{45}\text{Ca}^{2+}$ efflux from cultured SMC (31). Membrane depolarization does increase a slower component of $^{45}\text{Ca}^{2+}$ - $^{40}\text{Ca}^{2+}$ exchange (unpublished data). The stimulation by high K^+ or AII of the slower component of calcium exchange (which does not contribute significantly to rapid $^{45}\text{Ca}^{2+}$ release measured over 10-sec intervals as reported here) is markedly inhibited by removal of external Ca^{2+} (unpublished data) and probably reflects an increase in the Ca^{2+} permeability of the pericytoplasmic membrane. In contrast, the rapid release of $^{45}\text{Ca}^{2+}$ evoked by AII was not affected by the removal of external Ca^{2+} (Fig. 3). Therefore, an increase in Ca^{2+} influx is probably neither necessary, nor sufficient, for the stimulation of fast $^{45}\text{Ca}^{2+}$ release by AII. In fact, AII did not increase the initial rate of $^{45}\text{Ca}^{2+}$ influx (10–60 sec), indicating that the hormone released sequestered Ca^{2+} without detectably changing the Ca^{2+} permeability of the plasma membrane.

From the present results, it seems likely that AII increases *de novo* synthesis of both PtdOH and phosphoinositides and, concomitantly, hydrolysis of triphosphoinositide in cultured smooth muscle cells from rat aorta. Although statistically significant triphosphoinositide hydrolysis was not documented directly, there appeared to be a small initial decrease in triphosphoinositide evoked by AII (which presumably

was relatively small because of concomitant increase in triphosphoinositide synthesis), and Ins-P_3 generation was increased. The increase in Ins-P_3 could conceivably be due either to an increase in phospholipase C activity and consequent triphosphoinositide hydrolysis or to an increase in the availability of substrate, triphosphoinositide, to this enzyme. Against the latter possibility, however, is the fact that two other agents (adrenocorticotrophic hormone and insulin) that increase *de novo* phospholipid synthesis and triphosphoinositide levels (32, 33) do not appear to stimulate triphosphoinositide hydrolysis and have not been found to be associated with increases in Ins-P_3 generation (unpublished observations). In support of the former possibility, triphosphoinositide hydrolysis has been documented in at least two other tissues (24, 25) in response to AII. Obviously, the effects of AII on phospholipid metabolism will have to be examined in intact vascular tissue to further evaluate the role of triphosphoinositide hydrolysis in angiotensin action. Along these lines, it will be of interest to add purified Ins-P_3 to permeabilized SMC, as was recently described for hepatocytes (22, 23) and pancreatic acinar cells (21), and determine whether Ins-P_3 directly mobilizes sequestered calcium in vascular smooth muscle.

The increases in PtdIns and diphosphoinositide seem to be best explained by an increase in *de novo* synthesis of PtdOH and other phospholipids derived from PtdOH (34). This increase in *de novo* phospholipid synthesis in vascular smooth muscle is similar to that observed during the action of AII in adrenal glomerulosa tissue (26). The failure of the liver to respond to AII in a similar fashion with rapid increases in *de novo* synthesis of PtdIns and diphosphoinositide (32) may be related to the fact that, in this tissue, AII-induced glycogenolysis (35) apparently increases glucose release from the liver (by glucose-6-phosphatase action) and may diminish the flow of substrates down the glycolytic pathway because of inhibition of pyruvate dehydrogenase (36); in other tissues, such as vascular smooth muscle and adrenal cortex, AII-induced glycogenolysis may increase the availability of glycerol-3'-phosphate for subsequent synthesis of PtdOH.

Although it is likely that increases in triphosphoinositide hydrolysis are essential for increased Ins-P_3 generation, the increase in *de novo* phospholipid synthesis may nevertheless contribute importantly to the absolute amounts of Ins-P_3 generated. Obviously, increases in *de novo* synthesis of PtdIns and diphosphoinositide, as reported here, should result in an increase in triphosphoinositide synthesis and consequent availability for phospholipase C action. Cultured SMC from rat aorta, which are consistently responsive to angiotensin (13) and β -adrenergic agonists (31) for many passages, will likely be preeminent for further elucidation of Ca^{2+} regulation in vascular smooth muscle.

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