Vol. 156, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1988 Pages 240-245

NOREPINEPHRINE STIMULATES THE PRODUCTION OF INOSITOL TRISPHOSPHATE AND INOSITOL TETRAKISPHOSPHATE IN RAT AORTA

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Received September 6, 1988

SUMMARY Norepinephrine stimulated the rapid hydrolysis of [^{3}H]phosphatidylinositol-4,5-bisphosphate in rat aorta with a maximal decrease of 30% within 60 sec of stimulation. Levels of [^{3}H]phosphatidylinositol-4,5-bisphosphate returned to control by 5 min despite the continued presence of agonist. Hydrolysis of [^{3}H]phosphatidylinositol-4,5-bisphosphate occurred concurrently with the formation of inositol phosphates. Inositol-tris and tetrakisphosphate levels were increased within 30 sec of agonist stimulation. Increases in inositol phosphate levels due to agonist were dose-dependent with half-maximal activation at 1 μM norepinephrine.

The primary stimulus needed to promote contraction in smooth muscle is an increase in the cytosolic Ca^{2+} concentration (1). A number of agonists including norepinephrine, vasopressin and angiotensin II increase cytosolic Ca^{2+} through stimulation of Ca^{2+} release from intracellular stores as well as by promoting Ca^{2+} influx through putative receptor operated channels (1,2). The phosphoinositide-phospholipase C system appears to have an important role in the regulation of these events in vascular smooth muscle (3). Inositol-1,4,5-trisphosphate, derived from phospholipase C-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate, has been shown to elicit the release of Ca^{2+} from sarcoplasmic reticulum in permeabilized vascular smooth muscle cells (4,5,6) and muscle strips (7,8). Products of inositol-1,4,5-trisphosphate metabolism also appear to have an important role in regulation of cytosolic Ca^{2+} concentration. Inositol-1,3,4,5-tetrakisphosphate, generated from the phosphorylation of inositol-1,4,5-trisphosphate, may regulate Ca^{2+} influx (9).

In cultured smooth muscle cells obtained from rat aorta, both vasopressin and angiotensin II stimulated production of inositol-trisphosphate has been demonstrated to occur (10,11,12). There are limited studies in non-cultured vascular smooth muscle systems. In rabbit mesenteric artery, α_1-adrenergic amine-stimulation of inositol-trisphosphate has been shown to occur within 30 sec (5). In rat aorta, a system used to study regulation of vascular smooth muscle contraction, only production of inositol-monophosphate in response to agonist stimulation has been reported (13,14). Norepinephrine (NA)-stimulated an increase in inositol-monophosphate levels within 30 sec and a 20% decrease in phosphatidylinositol-4,5-bisphosphate levels within 10

Abbreviations: KHB, Krebs-Henseleit Buffer; GPI, glycerophosphorylinositol; $InsP_1$, inositol-monophosphate; $InsP_2$, inositol-bisphosphate; $InsP_3$, inositol trisphosphate; $InsP_4$, inositol-tetrakisphosphate; NA, norepinephrine; PIP_2, phosphatidylinositol-4,5-bisphosphate.

0006-291X/88 $1.50

sec (13). The inability to measure changes in inositol-trisphosphate levels despite the hydrolysis of phosphatidylinositol-4,5-bisphosphate could reflect a rapid phosphatase-mediated hydrolysis of inositol-trisphosphate as has been observed in numerous systems (15). The purpose of the present studies was to ascertain whether rapid changes in inositol-trisphosphate occurred in rat aorta in response to NA stimulation.

MATERIALS AND METHODS

Thoracic aortas from male rats (Sprague-Dawley) weighing 150-200 g were excised and cleansed of extraneous fatty tissue. Each aorta was cut into 4 or 6 pieces of equal sizes (approx. 1 cm in length). The tissue was labeled for 4-5 h with [^{3}H]inositol, as indicated, at 37°C in modified Krebs-Henseleit Buffer (KHB), equilibrated with 95% O_2/5% CO_2 consisting of 118 mM NaCl, 4.7 mM KCl, 3.0 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 0.5 mM EDTA, 25 mM $NaHCO_3$, 3.3 mM Hepes, 10 mM glucose and 20 mM Tris/HCl, (pH 7.4). Labeled tissue was transferred to test tubes containing one ml fresh KHB. LiCl and agonist were added for the indicated time. The incubations were terminated by the addition of 3 ml ice-cold methanol/chloroform (2:1 v/v) containing 0.1% HCl to the incubation tubes followed by the addition of 1 ml chloroform. The tissue was sonicated in the extraction medium for 1 h in an ice bath and extracted overnight at 5°C. Aqueous and lipid phases were separated by the addition of H_2O (2 ml). The upper phase was neutralized, applied to 0.5 ml Dowex-Formate resin slurry (AG1-X8) and poured into small columns. [^{3}H]Inositol was eluted with 40 ml H_2O followed by 4 ml each of 5 mM sodium tetraborate/60 mM ammonium formate to elute glycerophosphorylinositol (GPI) and 0.1 M formic acid containing 0.2, 0.4 and 1.0 M ammonium formate to elute inositol-monophosphate ($InsP_1$), inositol-bisphosphate ($InsP_2$), inositol-trisphosphate ($InsP_3$), respectively. In experiments in which inositol-tetrakisphosphate ($InsP_4$) was measured, $InsP_3$ was eluted with 0.1 M formic acid/0.8 M ammonium formate followed by the elution of $InsP_4$ with 0.1 M formic acid/1.0 M ammonium formate (16). Analysis of the elution profile for the individual inositol phosphates indicated that they eluted as separate and distinct peaks in regions corresponding to the elution of standards as documented (16). The lipid phase was evaporated in vacuo, resuspended in 50 µl chloroform and applied to silica gel plates for thin layer chromatographic separation of the labeled phosphoinositides. The solvent system used was methanol/chloroform/4N NH_4OH/water (90:90:9:19; v/v) (17). The labeled lipids were detected by fluorography using [^{3}H]Enhance. Lipids were scraped from the plates and radioactivity determined.

Norepinephrine (bitartrate salt) and phosphatidylinositol-4,5-bisphosphate were from Sigma Chemical Co. Myo-[2-^{3}H]-inositol (15 Ci/mmol) was obtained from American Radiolabeled Chem, Inc. and Amersham Corp. (19 Ci/mmol). AG1-X8 (formate form) was from Bio-Rad. Uniplate Silica-Gel HL plates were from Analtech. [^{3}H]Enhance was from New England Nuclear.

RESULTS AND DISCUSSION

The time course for production of inositol phosphates in response to NA stimulation in the presence of 25 mM LiCl is shown in Fig. 1. NA stimulated the rapid production of $InsP_1$, $InsP_2$ and $InsP_3$ within 60 sec. $InsP_3$ levels continued to increase slowly during a 15 min incubation. $InsP_1$ and $InsP_2$ increased at a greater rate than $InsP_3$. At 15 min, approximately five times as much labeled $InsP_1$ as $InsP_3$ was formed in response to agonist stimulation. These results demonstrate that rapid increases in $InsP_3$ do occur in rat aorta in response to NA stimulation.

In most tissues, at least two isomers of $InsP_3$ are generated in response to agonist stimulation. Ins-1,4,5-P_3 is the immediate product of phospholipase C-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate. An Ins-1,4,5-P_3-3-kinase mediates the phosphorylation of Ins-1,4,5-P_3 to form Ins-1,3,4,5-P_4 which is subsequently dephosphorylated to Ins-1,3,4-P_3 (18,19). The next series of experiments were designed to ascertain whether $InsP_4$ was generated in response to NA stimulation. Rat aorta were prelabeled with 150 µCi/ml

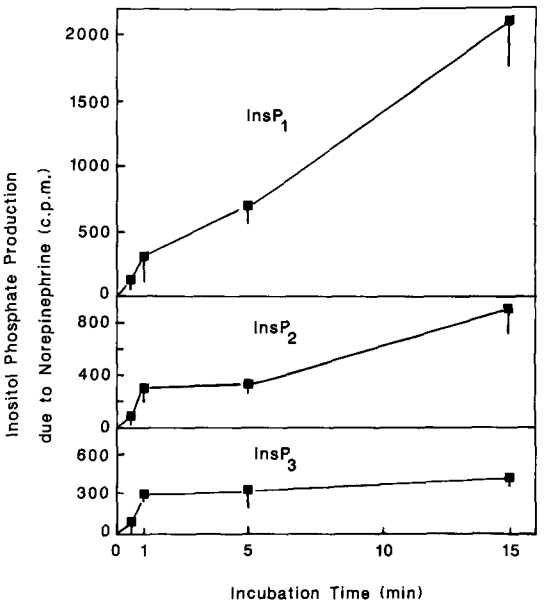

Figure 1 Norepinephrine Stimulated Production of Inositol Phosphates. Rat aortic strips were labeled with 45-55 μCi/ml [^{3}H]inositol for 4 h. Strips were transferred to fresh KHB to which 25 mM LiCl was added 2.5 min prior to the addition of agonist. Buffer control or norepinephrine (10 μM) was added for the indicated times. Each point represents the mean ± S.E.M for 4 experiments.

[^{3}H]inositol and the water soluble products of phosphoinositide hydrolysis were analyzed for production of $InsP_3$ and $InsP_4$. As shown in Fig. 2, NA stimulated an increase in the levels of $InsP_3$ and $InsP_4$ within 30 sec. The amount of $InsP_4$ produced was approximately half that of

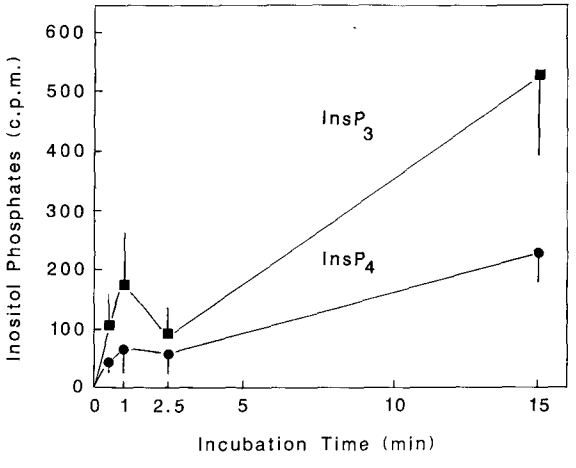

Figure 2 Norepinephrine Stimulated Production of Inositol Tris- and Tetrakisphosphate. Rat aortic strips were labeled for 4 h with 150 μCi/ml [^{3}H]inositol. The tissue was transferred to fresh KHB and subsequently incubated with or without 10 μM norepinephrine for the indicated times. 25 mM LiCl and 3 μM propranolol were present in the buffer. Each point represents the mean ± S.E.M. for 8 experiments.

TABLE I. Effect of LiCl on Basal and Norpinephrine Stimulated Inositol Phosphate Production

	(c.p.m.)			
Inositol Phosphates	Basal		Norepinephrine	
	-LiCl	+LiCl	-LiCl	+LiCl
$InsP_1$	150	260	230	430
$InsP_2$	140	300	290	420
$InsP_3$	160	260	230	360

Rat aortic strips were incubated for 3 h with 65 μCi/ml [^{3}H]inositol. The tissue was transferred to fresh KHB and incubated with or without 25 mM LiCl for 2.5 min followed by the addition of buffer control or 33 μM norepinephrine for 1 min.

$InsP_3$. Since $InsP_4$ is rapidly dephosphorylated to Ins-1,3,4-P_3, it is likely that the $InsP_3$ fraction contains both Ins-1,4,5-P_3 and Ins-1,3,4-P_3 (15).

The ability to detect $InsP_3$ and $InsP_4$ production in the present study as compared to other published studies may be a consequence of several factors. The amount of [^{3}H]inositol used to label the tissue was 45-150 μCi/ml as compared to 8-40 μCi/ml (13). Secondly, LiCl, at 25 mM was added to the incubation medium to inhibit phosphatase-mediated hydrolysis of $InsP_3$ (15). The effect of 25 mM LiCl on basal and NA-stimulated inositol phosphate production is shown in Table I. LiCl increased the basal levels of inositol phosphates by approximately 75%. LiCl did not markedly affect the distribution of label in the individual phosphates or the response to stimulation by NA. However, the increased radioactivity in the individual phosphates facilitated the detection of these compounds. LiCl was therefore used in subsequent experiments. Finally, it was observed that excessive handling of the labeled tissue resulted in an increase in basal $InsP_3$ levels. Under these conditions, it was difficult to consistently measure agonist-stimulated increases in $InsP_3$ levels. Thus, in all experiments, the incubation was terminated by the direct addition of ice-cold extraction medium rather than by removal of tissue from the medium as described in other studies (13,14).

The NA-stimulated production of inositol phosphates occurred concurrently with degradation of [^{3}H]phosphatidylinositol-4,5-bisphosphate (Fig. 3). A maximal decrease of 30% was observed within 60 sec of stimulation. After 60 sec, levels of [^{3}H]phosphatidylinositol-4,5-bisphosphate increased despite the continued presence of agonist. By 5 min, levels of [^{3}H]phosphatidylinositol-4,5-bisphosphate had returned to control. These results demonstrate that in rat aorta, as in many tissues including cultured vascular smooth muscle cells (10,11), the initial agonist-stimulated hydrolysis of [^{3}H]phosphatidylinositol-4,5-bisphosphate is followed by resynthesis. Whether the resynthesized lipid contributes to the continued agonist-stimulated production of $InsP_3$ remains to be determined.

Stimulation of inositol phosphate production due to NA was dose dependent (Fig. 4). Maximal accumulation of inositol phosphates was obtained with 100 μM NA. The EC_{50} was approximately 1 μM. Legan et al. (14) observed maximal stimulation of [^{3}H]inositol monophosphate production at 10 μM NA with an EC_{50} of 100 nM.

A slow increase in the levels of labeled glycerophosphorylinositol was also observed after 60 sec of incubation with NA (Fig. 5). An increase in glycerophorylinositol has also been

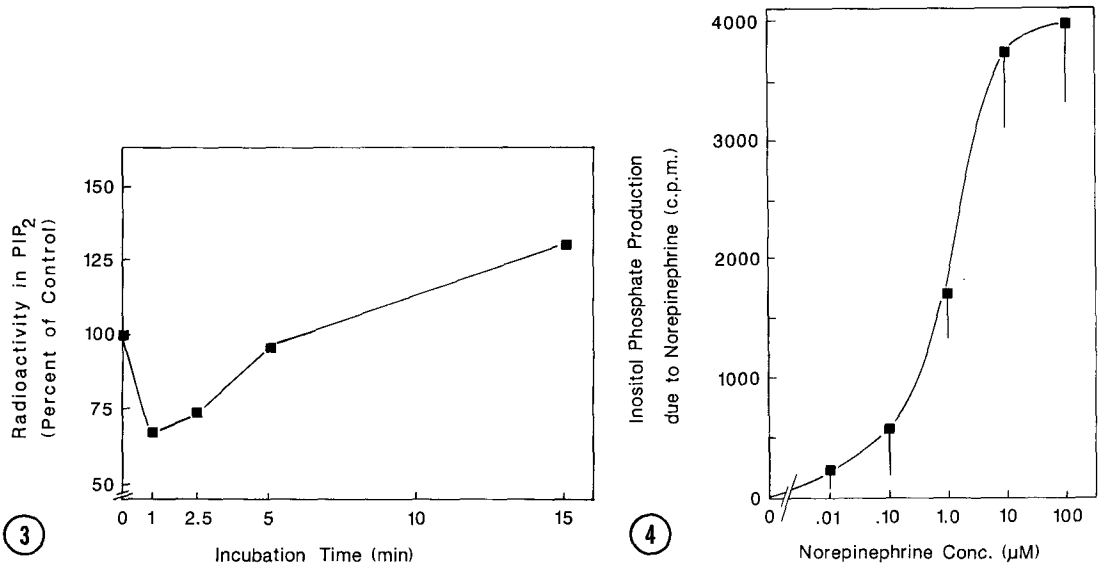

Figure 3 Time Course for Norepinephrine Stimulated Hydrolysis of [^{3}H]Phosphatidylinositol-4,5-Bisphosphate. Rat aortic strips were labeled with [^{3}H]inositol and incubated with or without 10 μM norepinephrine as described in the legend to Fig. 1. Changes in [^{3}H]phosphatidylinositol-4,5-bisphosphate (PIP_2) were analyzed by thin layer chromatography. Results are the mean of 6 experiments. The average S.E.M. was ± 27%. The average basal level of radioactivity in PIP_2 was 2000 c.p.m.

Figure 4 Dose Dependent Stimulation of Inositol Phosphate Production by Norepinephrine. Rat aortic strips were labeled with 45 μCi/ml [^{3}H]inositol for 4 h. The tissue was transferred to fresh KHB and 25 mM LiCl was added 2.5 min prior to the addition of agonist. Strips were subsequently incubated for 5 min with or without the indicated concentrations of norepinephrine. Each point represents the mean ± S.E.M. for 3-4 experiments.

observed in response to stimulation by vasopressin and angiotensin II in cultured rat aortic smooth muscle cells (10). The significance of this increase in glycerophosphorylinositol is presently unknown.

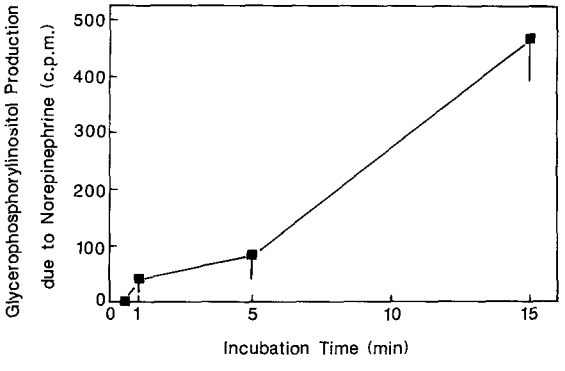

Figure 5 Norepinephrine Stimulated Production of Glycerophosphorylinositol. Rat aortic strips were labeled with 45-55 μCi/ml [^{3}H]inositol for 4 h. The tissue was transferred to fresh KHB to which 25 mM LiCl was added 2.5 min prior to the addition of agonist. Buffer control or norepinephrine (10 μM) was added for the indicated times. Each point represents the mean ± S.E.M. for 4 experiments.

Recent findings have shown that the agonist-stimulated accumulation of Ins-1,4,5-P_3 appears to be slower than the rise in intracellular free Ca^{2+} in a number of cells including GH_4C_1 cells (20), BC3H-1 cells (21) and 1321N1 cells (21). This apparent disparity in the kinetics of Ins-1,4,5-P_3 accumulation relative to the increase of Ca^{2+} may be due to 1) a localized effect of $InsP_3$ such that a small increase in Ins-1,4,5-P_3 is needed to elicit a maximal Ca^{2+} signal (20,21) or 2) multiple mechanisms may mediate the receptor-coupled increase in cytosolic Ca^{2+} (21,22). Characterization of the relationship of $InsP_3$ and $InsP_4$ production to Ca^{2+} elevation in rat aorta will allow further assessment of the role of the phosphoinositide pathway in Ca^{2+} elevation and vascular smooth muscle function.

ACKNOWLEDGEMENTS

This work was supported by an Established Investigator Award from the American Heart Association and with funds contributed from the AHA Florida Affiliate as well as by NIH DK-37007 and Lucille P. Markey Foundation.

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