

Angiotensin II-induced delayed stimulation of phospholipase C γ 1 requires activation of both phosphatidylinositol 3-kinase γ and tyrosine kinase in vascular myocytes

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Abstract

In vascular smooth muscles, angiotensin II (AII) has been reported to activate phospholipase C (PLC) and phosphatidylinositol 3-kinase (PI3K). We investigated the time-dependent effects of AII on both phosphatidylinositol 3,4,5-trisphosphate (PtdInsP3) and inositol phosphates (InsPs) accumulation in permeabilized microsomes from rat portal vein smooth muscle in comparison with those of noradrenaline (NA). AII stimulated an early production of PtdInsP3 (within 30 s) followed by a delayed production of InsPs (within 3–5 min), in contrast to NA which activated only a fast production of InsPs. The use of pharmacological inhibitors and antibodies raised against the PI3K and PLC isoforms expressed in portal vein smooth muscle showed that AII specifically activated PI3K γ and that this isoform was involved in the AII-induced stimulation of InsPs accumulation. NA-induced InsPs accumulation depended on PLC β 1 activation whereas AII-induced InsPs accumulation depended on PLC γ 1 activation. AII-induced PLC γ 1 activation required both tyrosine kinase and PI3K γ since genistein and tyrphostin B48 (inhibitors of tyrosine kinase), LY294002 and wortmannin (inhibitors of PI3K) and anti-PI3K γ antibody abolished AII-induced stimulation of InsPs accumulation. Increased tyrosine phosphorylation of PLC γ 1 was only detected for long-lasting applications of AII and was suppressed by genistein. These data indicate that activation of both PI3K γ and tyrosine kinase is a prerequisite for AII-induced stimulation of PLC γ 1 in vascular smooth muscle and suggest that the sequential activation of the three enzymes may be responsible for the slow and long-lasting contraction induced by AII.

Keywords: angiotensin II • phospholipase C gamma • phosphatidylinositol 3-kinase gamma • tyrosine-kinase • smooth muscle cells

Introduction

Angiotensin II (AII) has powerful effects on the mechanical and electrical activity of various smooth

muscle cells [1–3]. AII has been reported to activate different transduction pathways in vascular smooth muscle. One pathway involves activation of phospholipase C (PLC) inducing hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdInsP2) and generation of both inositol-1,4,5-trisphosphate (InsP3) and diacylglycerol, which promote the release of Ca²⁺ from intracellular stores and activation of protein kinase C, respectively [4]. Three families of mammalian PLC

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isoforms, PLC β , PLC γ and PLC δ , have been described on the basis of their molecular structure and mechanisms of regulation. PLC β has been shown to be activated by G α and G $\beta\gamma$ subunits of the heterotrimeric G proteins, while PLC γ is classically regulated by tyrosine phosphorylation [5]. AII has been shown to activate either PLC β 1 or PLC γ 1 in vascular myocytes [6, 7]. There is also evidence that PLC γ activity can be increased by phosphatidylinositol 3,4,5-trisphosphate (PtdInsP3) through binding to both pleckstrin homology domain and SH2 domains of the enzyme [8–10]. Another pathway activated by AII in vascular smooth muscle involves activation of phosphatidylinositol 3kinase (PI3K) which also uses PtdInsP2 to produce PtdInsP3. All class I PI3Ks are able to stimulate vascular L-type calcium channels [11] but only one isoform, PI3K γ , is involved in the stimulatory effects of AII on L-type calcium channels and increase in cytosolic calcium concentration ([Ca²⁺]_i) [12]. Interactions between these two pathways which use the same PtdInsP2 substrate may represent an example of signalling convergence leading to the physiological regulation of vascular contractility by AII.

In rat portal vein myocytes, it has been previously reported that AII activates the G α 13 β 1 γ 3 protein-coupled AT1A receptors [13] and that the β 1 γ 3 dimer of the G13 protein transduces the AII-induced stimulation of L-type calcium channels through activation of PI3K γ and protein kinase C [12, 14]. Ca²⁺ influx through calcium channels activates a Ca²⁺-induced Ca²⁺ release mechanism from the intracellular stores by opening of the ryanodine-sensitive Ca²⁺ release channels in the sarcoplasmic reticulum [15, 16]. Although this transduction coupling could be explained by the triggering of the AII-induced Ca²⁺ response, it remains to elucidate whether other cellular mechanisms could be involved in the vascular contractile effects of AII.

The purpose of the present study was to investigate the time-dependent effects of AII in comparison to those of noradrenaline (NA) on both PtdInsP3 and InsPs accumulations by using permeabilized microsomes from rat portal vein smooth muscle. Specific antibodies against PI3K and PLC isoforms expressed in portal vein smooth muscle were used in biochemical and functional experiments to identify the isoforms involved in the AII-induced transduction pathway. We show that AII activates a rapid production of PtdInsP3 *via* activa-

tion of PI3K γ and a delayed production of InsPs *via* activation of PLC γ 1 which needs the concomitant involvement of both PtdInsP3 and tyrosine kinase.

Material and methods

Portal vein microsomes

The investigation conforms with the European Community and French guiding principles in the care and use of animals. Authorizations to perform animal experiments were obtained from the Préfecture de la Gironde (A-33-063-003).

Wistar rats (140–160 g) were killed by cervical dislocation. The portal veins were cut into several pieces, homogenized in 20 mM Tris-HCl (pH 7.4, 4°C), 2 mM EGTA, 5 μ l inhibitor cocktail per g tissue wet weight and centrifuged at 170 x g for 10 min. The supernatant was submitted to ultra-centrifugation at 106,000 x g for 90 min at 4°C. The pellet composed of microsomes was resuspended in 20 mM Tris-HCl (pH 7.4, 4°C), 1 mM EGTA, 6 mM MgCl₂ and 1 mM CMP. Protein concentration was determined according to Bradford [17]. Phosphoinositides were labelled with 14 μ Ci [³H]-myo-inositol ([³H]-Ins) per mg of membranes and per ml of buffer, and 2 mM ATP [18]. After a 45-min incubation at 37°C, which is the time required for steady-state labelling, microsomes were centrifuged twice at 106,000 x g for 90 min to remove free [³H]-inositol. After dilution in 20 mM Tris-HCl (pH 7.4, 4°C), 2 mM EGTA and 5 μ l inhibitor cocktail, microsomes were divided into aliquots at a protein concentration of 200 μ g/ml/20,000 dpm and stored at –80°C for not more than 2 weeks.

Determination of [³H]-inositol phosphates and [³H]-phosphatidylinositol 3,4,5-tris-phosphate

Microsomes were permeabilized with 1 mM Na⁺ cholate, a method which allowed the uptake of substrate molecules but not the release of the endogenous enzymes and reaction products [19]. Microsomes were incubated at 37°C for 10 min in the presence of 15 mM LiCl, 50 μ M ATP, 100 nM GTP, 1 nM free Ca²⁺ buffered with 2 mM EGTA and 10 μ M propranolol to inhibit β -adrenoceptors [20]. Then, AII or noradrenaline (NA) were added at different concentrations and for different incu-

bation times. Reactions were terminated by adding $\text{CHCl}_3/\text{MeOH}/\text{HCl}$ (2/1/0.2, v/v/v). Extraction of [^3H]-InsPs and [^3H]-PtdInsPs from the samples were performed by adding 2M KCl/CHCl_3 (1/1, v/v) leading to the formation of two phases. The aqueous phase (methanolic) phase contained glycerophosphorylinositols (GPI), inositol and InsPs whereas the organic (chloroformic) phase contained inositol lipids. Radioactivity was quantified with a liquid-scintillation Packard 1500 Tri-Carb counter. Total InsPs accumulation was expressed as dpm / mg of protein. To avoid contamination with GPI, we checked that AII and NA did not affect GPI production and had no effect on phospholipase A activity [21]. Inositol lipid isomers contained in the chloroformic phase were separated by thin layer chromatography after pre-treatment of silica gel plates with 1 % potassium oxalate, 50 % ethanol and 1 mM EDTA. Phosphoinositides were separated using $\text{CHCl}_3/\text{MeOH}/\text{NH}_4^+/\text{H}_2\text{O}$ (70/100/15/25, v/v/v/v). PtdIns 3,4,5-P3, PtdIns 4,5-P2, PtdIns 4-P and PtdIns were eluted with the following Rf values : 0.10, 0.37, 0.50 and 0.63, respectively. Radioactivity was quantified by liquid-scintillation counting. PtdIns 3,4,5-P3 production was expressed as dpm / mg of protein.

Western blot analysis

Microsomal proteins were treated with Laemmli sample buffer containing 5% β -mercaptoethanol, boiled for 10 min and separated by SDS-PAGE (10% separating gel with 4% stacking gel). The resolved proteins were transferred to polyvinylidene difluoride (PVDF, Bio-Rad) membrane (1 h at 100 V). PVDF membrane was then blocked for 1 h with 3% BSA in PBS complemented with 0.1% Tween 20 (PBS-T) and then incubated overnight with the primary anti-PLC isoform antibodies at 1 μg / ml. After extensive washes in PBS-T, membranes were incubated for 2 h with a 0.4 μg / ml dilution of peroxidase-coupled anti-rabbit or anti-goat IgG in PBS-T complemented with 3% BSA. Specific antigen detection was performed using the Bio-Rad Opti 4CN Substrate Kit. Gels were analyzed with KDS1D 2.0 software (Kodak Digital Science, Paris, France).

Immunoprecipitation

Microsomes were lysed in a buffer containing 1% NP-40, 1 mM orthovanadate, protease inhibitor and phos-

phatase inhibitor cocktail. Samples were precleared with 100 μl / ml protein A-sepharose beads and immunoprecipitation was carried out with anti-PLC γ 1 antibody at 10 μg / ml preadsorbed on protein A-sepharose beads. The protein A-sepharose-bound immune complex was washed twice in buffer containing 1% NP-40 and once in buffer without detergent. Pellets from the immunoprecipitations were treated with Laemmli sample buffer for SDS-polyacrylamide gel electrophoresis and analyzed by immunoblot with anti-phosphotyrosine (PY 20) antibody at 1 $\mu\text{g}/\text{ml}$.

Measurement of cytosolic Ca^{2+}

Isolated myocytes from rat portal vein were obtained by enzymatic dispersion, as described previously [13]. Cells were seeded at density of about 10^3 cells / mm^2 on glass slides in physiological solution and used within 24 h. Measurements of [Ca^{2+}] $_i$ were performed with an indo-1 set-up described elsewhere [15]. Briefly, cells were preloaded with the membrane permeable indo-1 AM (1 μM) for 30 min at room temperature. [Ca^{2+}] $_i$ was estimated from the 405/480 nm fluorescence ratio, by using a calibration determined within cells. When intracellular infusion of antibodies was needed, 50 μM indo-1 was added to the pipette solution and both compounds entered the cells after establishment of the whole-cell recording mode, as previously reported [15]. The normal physiological solution contained (in mM): 130 NaCl, 5.6 KCl, 1 MgCl_2 , 1.7 CaCl_2 , 11 glucose and 10 HEPES, pH 7.4. The basic pipette solution contained (in mM) : CsCl 130, HEPES 10, pH 7.3 with CsOH.

Contraction experiments

Isometric contractions of longitudinal strips from rat portal vein were recorded in an experimental chamber described previously [1, 22] by means of a highly sensitive isometric force transducer (Akers 801 AME, Norten, Norway). The circulation solution was similar to that used for measurements made on isolated myocytes and was maintained at $35 \pm 1^\circ\text{C}$.

Chemicals and drugs

Angiotensin II was from Neosystem Laboratories (Strasbourg, France). Myo-[2- ^3H (N)]-inositol and [^3H]-

PtdIns isomers were from NEN (Dupont de Nemours, France). Genistein, daidzein, U73122 and U73343 were from Biomol (Palo Alto, CA). Tyrphostin B48 and A1, LY294002, Indo-1 and Indo-1 AM were from Calbiochem (Meudon, France). [1-*O*-stearoyl-2-*O*-arachidonoyl-sn-glycer-3-yl-D-myo-inositol-3,4,5-trisphosphate] (PtdInsP3) was from Alexis Biochemicals (Paris, France). Rabbit polyclonal anti-PLC β 1 (sc-205), anti-PLC β 2 (sc-9018), anti-PI3K β (sc-7175), anti-PI3K δ (sc-7176), anti-PI3K γ (sc-7177) and goat polyclonal anti-PI3K α antibody (sc-1331) were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-PLC γ 1 (P-12220), anti-PLC δ 1 (P-33820) and anti-phosphotyrosine PY20 (P-11230) antibodies were from Transduction Laboratories (BD Sciences, Le Pont de Claix, France). Solvents were from Prolabo (Fontenay s/Bois, France). All other products were from Sigma (Saint Quentin Fallavier, France).

Data analysis

Data are expressed as means \pm s.e. mean. Significance was tested by means of Student's *t*-test. $p < 0.05$ was considered as significant.

Results

Contractile responses to angiotensin II and noradrenaline in portal vein smooth muscle

As illustrated in Fig. 1A, contractions evoked by angiotensin II (AII) and noradrenaline (NA) were characterized by an increase in basal tone associated with an increase in frequency of reduced spontaneous contractions. After withdrawal of the mediators, both spontaneous contractions and basal tone returned progressively to control. However, the time course of AII-induced contractions strongly differed from that of NA-induced contractions, i.e. the delay between agonist application and half-maximal contraction was 2.41 ± 0.22 min for AII and 0.18 ± 0.14 min for NA ($n = 15$), respectively and recovery after agonist removal was 8.70 ± 0.78 min for AII and 3.52 ± 0.47 min for NA ($n = 15$), respectively (Fig. 1B).

Effects of various pharmacological inhibitors of predicted enzymes involved in transduction path-

ways were tested on the peak amplitude of AII- and NA-induced contractions. As shown in Fig. 1 C–D, losartan (a non-peptidic antagonist of AT1 receptors) selectively inhibited the AII-induced contraction with a concentration corresponding to half-maximal inhibition (IC_{50}) of 11.5 ± 0.8 nM ($n = 5$) whereas prazosin (a α_1 adrenoceptor antagonist) selectively inhibited the NA-induced contraction with an IC_{50} value of 4.5 ± 1.5 nM ($n = 5$). Interestingly, inhibitors of PI3K (LY294002) and tyrosine kinase (genistein and tyrphostin B48) inhibited in a concentration-dependent manner the AII-induced contractions (Fig. 1C) with IC_{50} values of 5.5 ± 0.7 μ M for LY297002, 7.7 ± 0.9 μ M for tyrphostin B48 and 10.4 ± 1.1 μ M for genistein, respectively ($n = 4$) whereas they were ineffective on the NA-induced contractions (Fig. 1D; $n = 4$). At low concentration (100 nM), wortmannin (a PI3K inhibitor) suppressed the AII-induced contraction ($n = 4$) but also reduced the NA-induced contraction ($n = 4$), an effect which could be related to inhibition of myosin light chain kinase [23]. Similarly, U73122 (a PLC inhibitor) which also blocked Ca^{2+} channel activity [24] inhibited both NA- and AII-induced contractions (Fig. 1 C–D; $n = 4$). It can be noted that U73343 (the inactive analog of U73122) had no effect on the NA-induced contraction whereas it reduced the AII-induced contraction, an effect probably related to its inhibitory action on L-type Ca^{2+} channels [24]. Since AII-induced contractions were affected by inhibitors of PLC, PI3K and tyrosine kinase, these data suggest the possibility of a signalling convergence to account for the long-lasting vascular contraction evoked by AII.

Time course of phosphatidylinositol 3,4,5-trisphosphate and inositol phosphate accumulation induced by AII and NA

We investigated the time course of phosphatidylinositol 3,4,5-trisphosphate (PtdInsP3) and inositol phosphates (InsPs) accumulation induced by AII in portal vein smooth muscle microsomes permeabilized by application of 1 mM Na^+ cholate [19]. AII (10 nM) induced a time-dependent accumulation of PtdInsP3 which reached a peak value at 30 s and remained elevated up to 5 min (Fig. 2A). The concentration-dependent curve established for AII applications of 30 s (Fig. 2B) revealed that the half-maximal

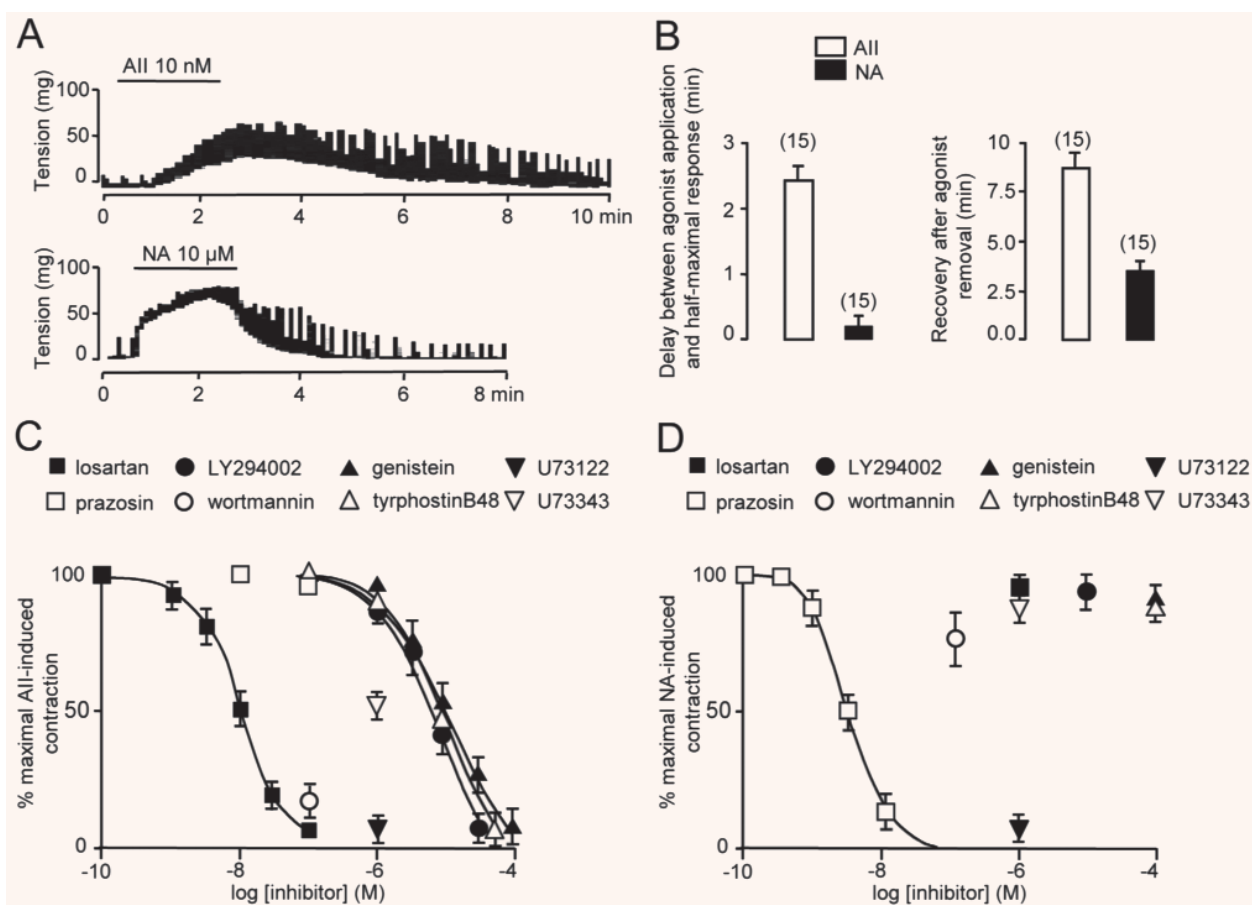
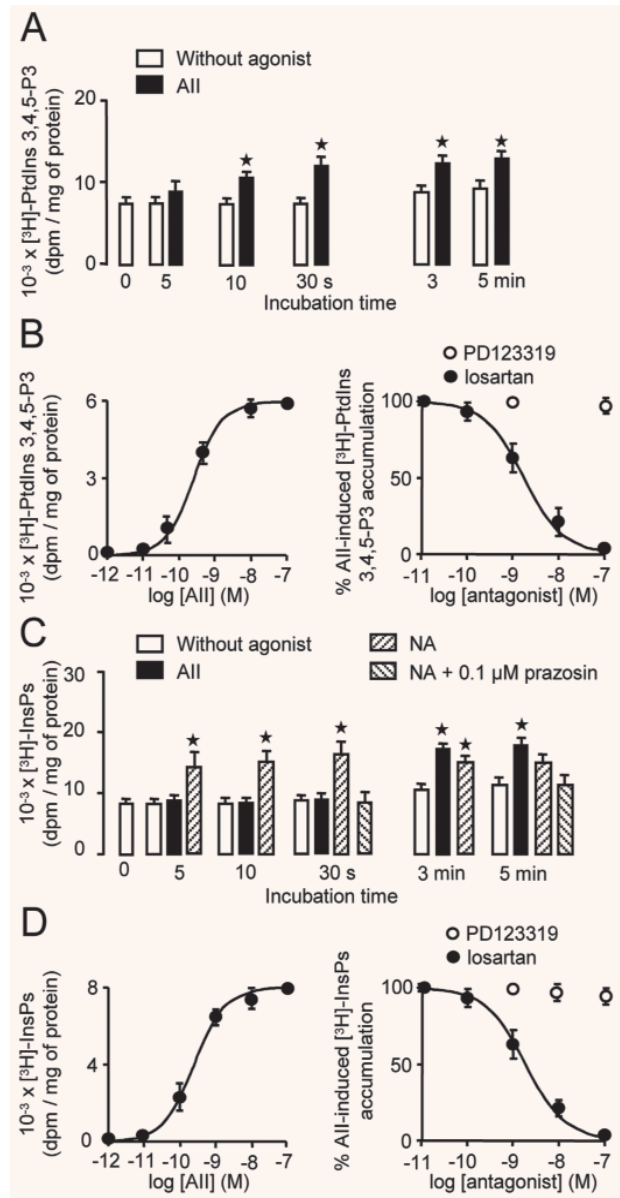


Fig. 1 Contractile responses to angiotensin II (AII) and noradrenaline (NA) in intact strips from rat portal vein. (**A**–**B**) Typical recordings of contraction to 10 nM AII (**A**) or 10 μM NA. (**B**) Kinetic parameters for AII- and NA-induced contractions shown as the delay between agonist application and half-maximal response and the recovery after agonist removal. Data are means \pm s.e. mean with the number of experiments indicated in parentheses. (**C**) Effects of losartan (AT1 receptor antagonist), prazosin (α_1 adrenoceptor antagonist), LY294002 and wortmannin (PI3K inhibitors), genistein and tyrphostin B48 (tyrosine kinase inhibitors), U73122 and U73343 (PLC inhibitor and inactive analog, respectively) applied for 10 min on AII-induced contractions. (**D**) Effects of the same pharmacological molecules on NA-induced contractions. Contractions are expressed as a percentage of the maximal contractile responses induced by 10 nM AII or 10 μM NA and are shown as means \pm s.e. mean of four or five experiments.

response for PtdInsP3 accumulation was obtained at 0.3 ± 0.9 nM AII and the maximal effect at 0.1 μM AII ($n = 4$). When PtdInsP3 accumulation was determined in the presence of increasing concentrations of losartan (a non-peptidic antagonist of AT1 receptors), the AII-induced stimulation was progressively inhibited (Fig. 2B) with an IC_{50} value of 1.2 ± 1.0 nM ($n = 4$). In contrast, PD123319 (a non-peptidic antagonist of AT2 receptors) had no effect on the AII-induced stimulation of PtdInsP3 accumulation (Fig. 2B). It could be noted that during application of NA within the same time no significant PtdInsP3 accumulation was detected ($n = 4$).

AII (10 nM) applied within 30 s had no significant effect on InsPs accumulation whereas NA (10 μM) was maximally effective within 5–30 s (Fig. 2C). Interestingly, longer applications of AII induced a time-dependent increase in InsPs accumulation which was detected at 2–3 min and remained elevated within 5–10 min of AII application (Fig. 2C). NA-induced stimulation of InsPs accumulation was suppressed by application of 0.1 μM prazosin. HPLC analysis of InsPs produced upon AII stimulation established that AII stimulated phosphatidylinositol 4,5-P2 (PtdInsP2) breakdown to give Ins 1,4,5-P3 but also highly-phos-

Fig. 2 [^3H]-PtdIns 3,4,5-P3 and [^3H]-InsPs accumulation in rat portal vein smooth muscle induced by AII and NA. (A) Time-course of [^3H]-PtdIns 3,4,5-P3 accumulation induced by AII in permeabilized microsomes from rat portal vein smooth muscle. [^3H]-inositol-labelled permeabilized microsomes were incubated for 10 min with 10 mM LiCl before addition of 10 nM AII. Stimulations were stopped at times indicated and [^3H]-PtdIns 3,4,5-P3 was separated. PtdIns 3,4,5-P3 accumulations are expressed as dpm/mg of protein and are means \pm s.e. mean of four experiments. (B) [^3H]-PtdIns 3,4,5-P3 accumulation (after subtraction of basal production) in the presence of increasing concentrations of AII for 30 s and effects of increasing concentrations of losartan (filled points) or PD123319 (open points) on [^3H]-PtdIns 3,4,5-P3 accumulation induced by 10 nM AII for 30 s. Data are expressed as dpm / mg of protein and as a percentage of net [^3H]-PtdIns 3,4,5-P3 accumulation induced by 10 nM AII; they are means \pm s.e. mean of four experiments. In our experimental conditions, basal PI3K activity was 2.5 ± 0.2 pmol PtdInsP3 / min/mg protein (n = 12). (C) Time course of [^3H]-InsPs accumulation induced by 10 nM AII and 10 μM noradrenaline (NA), and effects of 0.1 μM prazosin (applied for 5 min) on NA-induced [^3H]-InsPs accumulation at 30 s and 5 min. Total InsPs accumulations are expressed as dpm/mg of protein and are means \pm s.e. mean of four experiments. (D) [^3H]-InsPs accumulation (after subtraction of basal production) in the presence of increasing concentrations of AII for 5 min and effects of increasing concentrations of losartan (filled points) or PD123319 (open points) on [^3H]-InsPs accumulation induced by 10 nM AII for 5 min. Data are expressed as dpm/mg of protein and as a percentage of net [^3H]-InsPs accumulation induced by 10 μM NA; they are means \pm s.e. mean of four experiments. In our experimental conditions, basal PLC activity was 3.9 ± 0.9 pmol InsPs / min / mg protein (n = 8). *, values significantly different from those obtained in basal conditions (p<0.05).



phorylated InsPs, such as Ins1,3,4,5-P4, Ins1,3,4,5,6-P5 and InsP6 [25]. The concentration-dependent curve established for AII applications of 5 min (Fig. 2D) revealed that the half-maximal response for InsPs accumulation was obtained at 0.2 ± 1.1 nM AII and the maximal effect at 0.1 μM AII (n = 4). When InsPs accumulation was determined in the presence of increasing concentrations of losartan, the AII-induced stimulation of InsPs accumulation was inhibited with an IC50 value of 2.2 ± 1.1 nM (n = 4), whereas PD123319 had no effect (Fig. 2D). Taken together, these results show that in permeabilized microsomes from portal vein smooth muscle, AII *via* activation of AT1

receptors induces an early increase in PtdInsP3 accumulation followed by a delayed stimulation of InsPs accumulation. This is in contrast with the single stimulation of InsPs accumulation induced by NA in the same preparation.

PI3K isoforms involved in AII-induced stimulation of PtdInsP3 accumulation

Application of wortmannin and LY294002 (pharmacological inhibitors of PI3K) inhibited in a concentration-dependent manner the AII-stimulated PtdInsP3 accumulation with a maximal effect

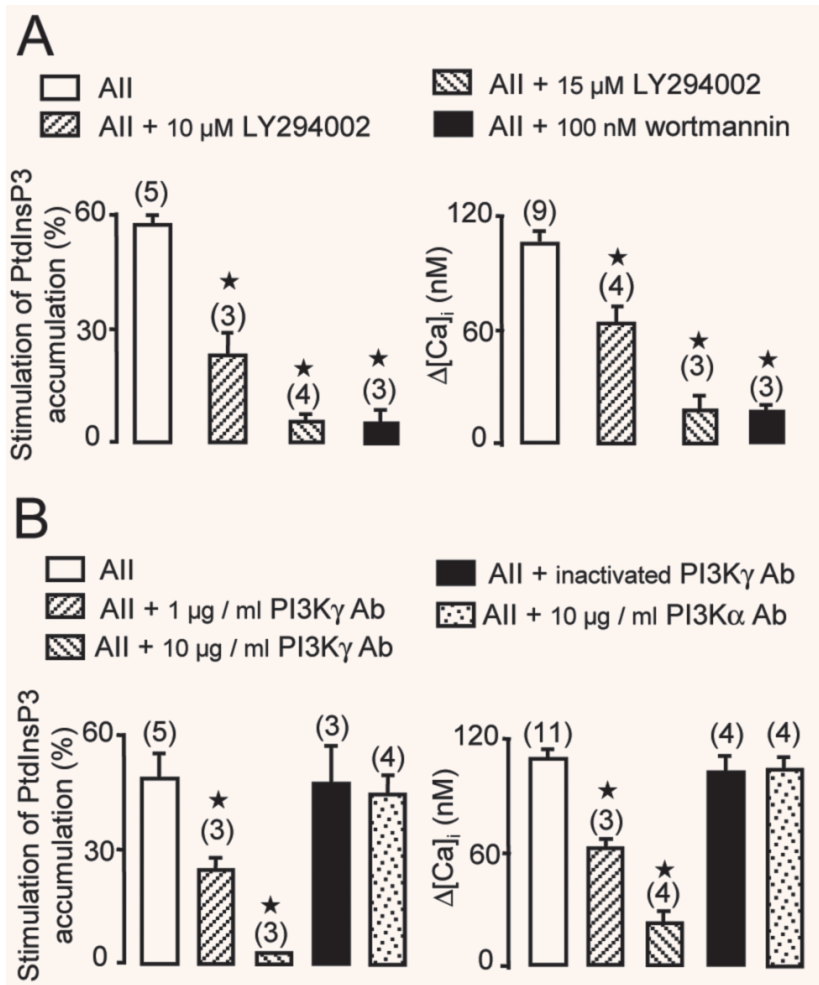
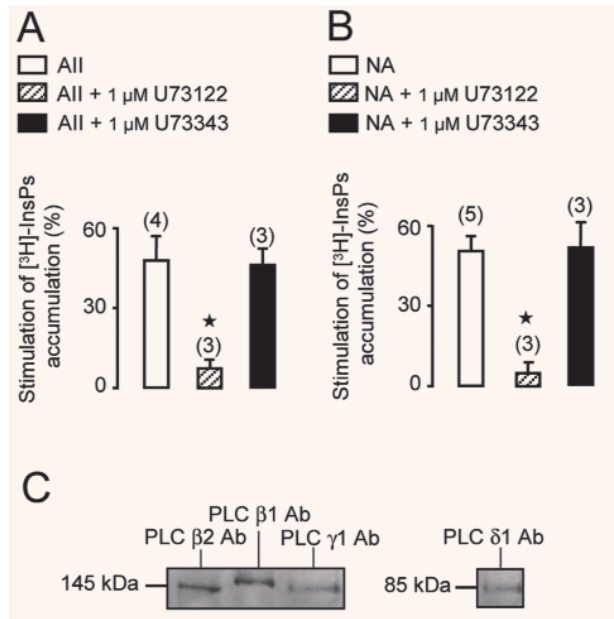


Fig. 3 Effects of inhibitors of PI3K on the stimulation of [³H]-PtdInsP3 accumulation induced by 10 nM AII for 30 s and on AII-induced Ca²⁺ responses. **(A)** Effects of LY294002 and wortmannin on the stimulation of [³H]-PtdInsP3 accumulation and Ca²⁺ responses evoked by 10 nM AII. **(B)** Effects of increasing concentrations of anti-PI3Kγ antibody, 10 μg/ml anti-PI3Kγ antibody or 10 μg/ml anti-PI3Kα antibody pre-incubated with 10 μg/ml of its antigen peptide on the stimulation of [³H]-PtdInsP3 accumulation and Ca²⁺ responses evoked by 10 nM AII. Data are means ± s.e. mean with the number of experiments indicated in parentheses. Basal PI3K activity was 2.6 ± 0.2 pmol PtdInsP3 /min/mg protein (n = 9). *, values significantly different from those obtained in control conditions (p<0.05).

obtained at 100 nM and 15 μM, respectively (Fig. 3A). Similar concentrations of PI3K inhibitors also inhibited the AII-induced Ca²⁺ responses (Fig. 3A). Use of antibodies directed against signalling enzymes have been reported to be useful for identifying neuromediator-activated transduction pathways [22]. As we have previously reported by Western blotting that portal vein smooth muscle expresses only PI3Kα and PI3Kγ [11], we investigated which types of PI3K were stimulated by AII by pre-incubation of the permeabilized microsomes (during 2 h at 4°C) with antibodies raised against p110α (anti-PI3Kα antibody) or p110γ (anti-PI3Kγ antibody). These antibodies are believed to be specific as they interact only with the corresponding purified recombinant PI3K isoform [11] and selectively inhibit the stimulatory effects of the corresponding purified recombinant enzyme on the L-type Ca²⁺ channel current [12]. In the latter case, antibodies were infused into the cells through the patch-clamp

pipette. As illustrated in figure 3B, PtdInsP3 accumulation was measured after a 30-s incubation period with 10 nM AII in the absence or in the presence of the anti-PI3K isoform antibodies. Application of 10 μg / ml anti-PI3Kα antibody had no significant effect on the AII-induced stimulation of PtdInsP3 accumulation (Fig. 3B) whereas anti-PI3Kγ antibody inhibited in a concentration-dependent manner the AII-induced stimulation of PtdInsP3 production (Fig. 3B). Maximal inhibition was obtained with 10 μg / ml anti-PI3Kγ antibody. Inactivation of the anti-PI3Kγ antibody by a pre-incubation with its epitope peptide (10 μg / ml) suppressed its inhibitory effect on the AII-induced stimulation of PtdInsP3 accumulation. Similar effects of the anti-PI3K isoform antibodies were obtained on the AII-induced Ca²⁺ responses (Fig. 3B). These results show that AII-induced activation of PI3Kγ is responsible for the early production of PtdInsP3 and the triggering of Ca²⁺ responses in portal vein myocytes.

Fig. 4 Effects of PLC inhibitors on AII- and NA-induced stimulation of [³H]-InsPs accumulation and expression of PLC isoforms in rat portal vein smooth muscle. (A-B) Effects of U73122 and U73343 on the stimulation of [³H]-InsPs accumulation induced by 10 nM AII for 5 min (A) and 10 μM NA for 10 s (B). Data are means ± s.e. mean with the number of experiments indicated in parentheses. Basal PLC activity was 3.1 ± 0.3 pmol InsPs / min / mg protein (n = 11). *, values significantly different from those obtained in control conditions (p < 0.05). (C) Microsomes of rat portal vein smooth muscle (50 μg of protein/vial) were separated on SDS-PAGE and analyzed by Western blot with anti-PLCβ1, anti-PLCβ2, anti-PLCγ1 and anti-PLCδ1 antibodies. Molecular weights estimated from molecular weight standards electrophoresed on the same gels. Similar results were obtained in five separate experiments.



PLC isoforms involved in AII- and NA-induced stimulation of InsPs accumulation

Application of U73122 (a pharmacological inhibitor of PLC) inhibited both AII- and NA-induced stimulation of InsPs accumulation (Fig. 4 A–B). It could be noted that U73343 (the inactive analog of U73122) had no effect on the mediators-stimulated InsPs accumulation. Then, we identified by Western blotting the PLC isoforms expressed in microsomes from portal vein smooth muscle *i.e.* PLCβ1 and β2, PLCγ1 and PLCδ1 (Fig. 4C). Incubation of permeabilized microsomes with the anti-PLCγ1 antibody inhibited in a concentration-dependent manner the AII-induced stimulation of InsPs accumulation with a maximal effect obtained at 10 μg / ml anti-PLCγ1 antibody (Fig. 5A). Unspecific effect of the antibody is unlikely because the boiled antibody (95°C for 30 min) failed to decrease the AII-induced stimulation of InsPs accumulation. Another control is provided in Fig. 5B, showing that the same antibody largely inhibited the AII-induced Ca²⁺ response. In addition, incubation with 10 μg / ml anti-PLCβ1, anti- PLCβ2 or anti-PLCδ1 antibody had no effect on both AII-induced InsPs accumulation (Fig. 5A) and AII-induced Ca²⁺ responses (Fig. 5B). In contrast, both NA-induced stimulation of InsPs accumulation (Fig. 6A) and NA-induced Ca²⁺ responses (Fig. 6B) were concentration-dependently inhibited by the anti-PLCβ1 antibody whereas they were not significantly affected by the boiled anti-PLCβ1 antibody and the other anti-PLC isoform antibodies.

Incubation of permeabilized microsomes with LY294002 and wortmannin (inhibitors of PI3K activity) inhibited in a concentration-dependent manner the AII-induced stimulation of InsPs accumulation (Fig. 7A) but had no significant effects on the NA-induced stimulation of InsPs accumulation (Fig. 7B). Moreover, inhibition of AII-induced stimulation of InsPs accumulation was also obtained with the anti-PI3Kγ antibody (with a maximal effect obtained at 10 μg / ml) but not with the anti-PI3Kα antibody (Fig. 7C), suggesting that PI3Kγ was involved in this transduction. However, addition of PtdInsP3 (10 μM) to the incubation medium was not able to stimulate InsPs accumulation by its own, indicating that PtdInsP3 alone could not reproduce the AII-induced effects on InsPs accumulation (Fig. 7D). As expected, PtdInsP3 was able to compensate PI3K inhibition by LY294002 since similar AII-induced InsPs accumulations were obtained in the presence of AII alone or in the presence of AII + LY294002 + PtdInsP3 (Fig. 7D). These results suggest that PtdInsP3 is necessary for AII-mediated PLCγ1 activation.

Involvement of tyrosine kinase in AII-induced stimulation of InsPs accumulation

It is generally accepted that activation of PLCγ1 can be obtained in the absence or in coordination with tyrosine kinase phosphorylation. To show that AII-induced activation of PLCγ1 required the involve-

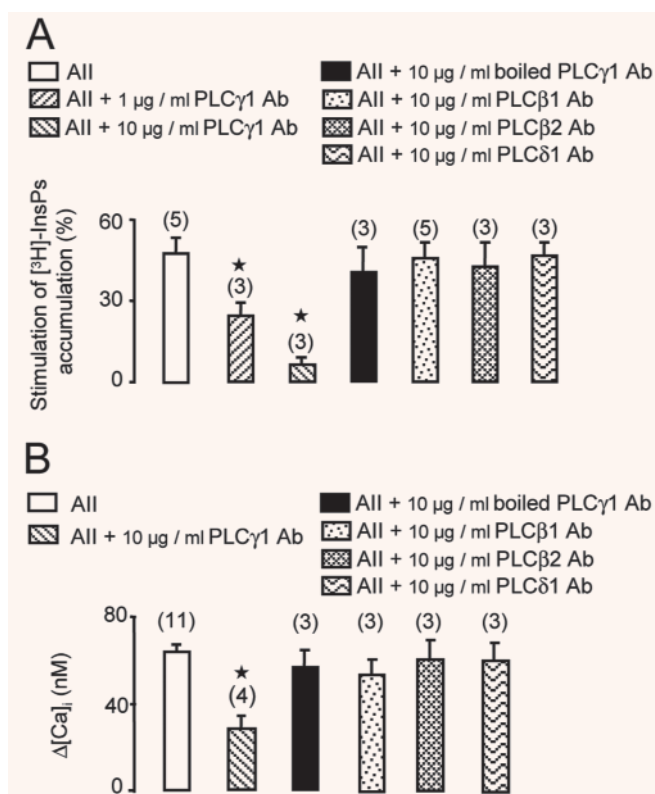


Fig. 5 Effects of the anti-PLC isoform antibodies on AII-induced stimulation of $[^3\text{H}]\text{-InsPs}$ accumulation and AII-induced Ca^{2+} response. **(A)** Effects of increasing concentrations of anti-PLC γ 1 antibody, 10 $\mu\text{g/ml}$ boiled anti-PLC γ 1 antibody, 10 $\mu\text{g/ml}$ anti-PLC β 1, anti-PLC β 2 and anti-PLC δ 1 antibody on the stimulation of $[^3\text{H}]\text{-InsPs}$ accumulation induced by 10 nM AII for 5 min. Basal PLC activity was 3.2 ± 0.4 pmol InsPs/min/mg protein ($n = 9$). **(B)** Effects of the same anti-PLC isoform antibodies on the Ca^{2+} responses evoked by 10 nM AII in the external and internal presence of 10 μM ryanodine for 5 min to block ryanodine channels. The cells were loaded with Indo-1 and patch-clamped. *, values significantly different from those obtained in control conditions ($p < 0.05$).

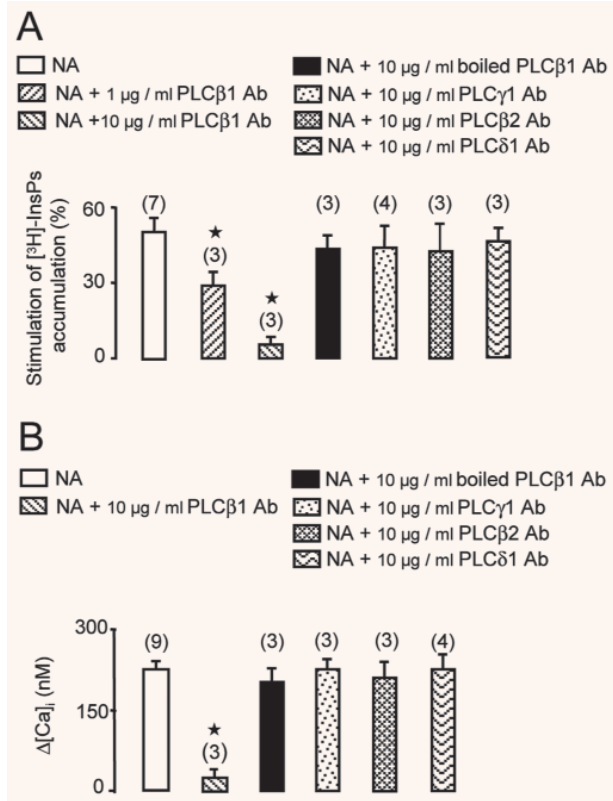
ment of a tyrosine kinase, experiments were performed in the presence of tyrosine-kinase inhibitors, genistein and tyrphostin B48. As shown in Fig. 7E, genistein and tyrphostin B48 inhibited in a concentration-dependent manner the AII-induced stimulation of InsPs accumulation. In the presence of the inactive compounds, daidzein or tyrphostin A1, the AII-induced stimulation of InsPs accumulation was similar to that obtained in control conditions (Fig. 7E). It was noted that the NA-stimulated InsPs accumulation was not significantly affected by genistein and tyrphostin B48 (Fig. 7B). To detect an increased phosphorylation of PLC γ 1 by AII, PLC γ 1 was immunoprecipitated and tyrosine-phosphorylated proteins were detected with the anti-PY20 antibody. Fig. 8 shows that tyrosine-phosphorylated PLC γ 1 was detected under control conditions and that the amount of tyrosine-phosphorylated PLC γ 1 was not significantly affected in the presence of AII for 30 s but was strongly increased in the presence of AII for 5 min. In addition, a pre-treatment with 30 μM genistein for 5 min suppressed the increase in PLC γ 1 phosphorylation induced by AII (Fig. 8). These results support the idea that phosphorylation of PLC γ 1 by AII was essential for activation of PLC γ 1.

Discussion

In this study, we show that in permeabilized microsomes from rat portal vein smooth muscle, AII activates an early production of PtdInsP3 *via* activation of PI3K γ , followed by a delayed production of InsPs *via* activation of PLC γ 1 which requires the concomitant involvement of both PI3K γ and tyrosine kinase.

Our results reveal that AII stimulated PtdInsP3 accumulation with a maximal production obtained within 10–30 s; however, PtdInsP3 remained elevated up to 5 min. This time course was noticeably different from that of InsPs accumulation which was only detected within 2–3 min and remained elevated within 5–10 min. Although AII-induced PtdInsP3 production has been previously reported in coronary artery [26, 27], the PI3K isoforms involved in AII-induced PtdInsP3 production have not been identified. Since PI3K α and PI3K γ are expressed in rat portal vein smooth muscle [11, 12], incubation with specific anti-PI3K α or anti-PI3K γ antibody revealed that both PtdInsP3 and InsPs productions induced by AII only required the PI3K γ isoform. Moreover, AII-stimulated PtdInsP3 and

Fig. 6 Effects of the anti-PLC isoform antibodies on NA-induced stimulation of [³H]-InsPs accumulation and NA-induced Ca²⁺ response. **(A)** Effects of increasing concentrations of anti-PLCβ1 antibody, 10 μg/ml boiled anti-PLCβ1 antibody, 10 μg/ml anti-PLCγ1, anti-PLCβ2 and anti-PLCδ1 antibody on the stimulation of [³H]-InsPs accumulation induced by 10 μM NA for 10 s. Basal PLC activity was 3.1 ± 0.1 pmol InsPs / min/mg protein (n = 21). **(B)** Effects of the same anti-PLC isoform antibodies on the Ca²⁺ responses evoked by 10 μM NA. The cells were loaded with Indo-1 and patch-clamped. Data are means ± s.e. mean with the number of experiments indicated in parentheses. *, values significantly different from those obtained in control conditions (p<0.05).



InsPs accumulations were dependent on activation of AT1 receptors, as they were selectively inhibited by losartan and not by PD123319. These results are consistent with our previous data showing that AII-induced PI3Kγ activation is responsible for the stimulation of vascular L-type Ca²⁺ channels [12].

Although expression of both PLCβ and PLCγ isoforms have been detected in different smooth muscles [28–30], the nature of the isoforms involved in the AII-induced production of InsPs remains controversial. Either PLCβ1 or PLCγ1 isoforms have been reported to be involved in AII-induced signalling pathway [6, 7, 31, 32] but the time-dependent activation of these enzymes has not been studied. In rat portal vein permeabilized microsomes, we revealed that AII did not stimulate an early InsPs accumulation (within 5–30 s) in contrast to that obtained with NA in the same preparations. However, a significant increase in InsPs accumulation was detected after several min of AII application (within 3–10 min). The possibility that different PLC isoforms could be activated by AII and NA was studied by using specific antibodies against the PLC isoforms expressed in portal vein smooth muscle. We showed that only PLCγ1 was

involved in the AII-induced production of InsPs, in contrast with NA which only activated PLCβ1. Specificity of the PLC isoform antibodies was supported by results showing that the anti-PLCγ1 antibody inhibited both AII-induced stimulation of InsPs accumulation and AII-induced Ca²⁺ response but not NA-induced responses. Similarly, the anti-PLCβ1 antibody inhibited both NA-induced stimulation of InsPs accumulation and NA-induced Ca²⁺ response but not AII-induced responses. These results are in good agreement with previous data showing that (1) α1-adrenoceptors are coupled to the Gq protein [33] and the α-subunits of the Gq protein family selectively activate PLCβ1 [34]; (2) AT1 receptors are coupled to the G13 protein and the transduction pathway involves the Gβγ dimer [13]. We found that AII-stimulated phosphoinositide breakdown was dependent on tyrosine kinase activity, because the tyrosine kinase inhibitors genistein and tyrphostine B48 inhibited in a concentration-dependent manner the AII-induced stimulation of InsPs accumulation and the AII-induced contractile response. This is in contrast with the absence of effects of genistein and tyrphostine B48 on both NA-induced stimulation of

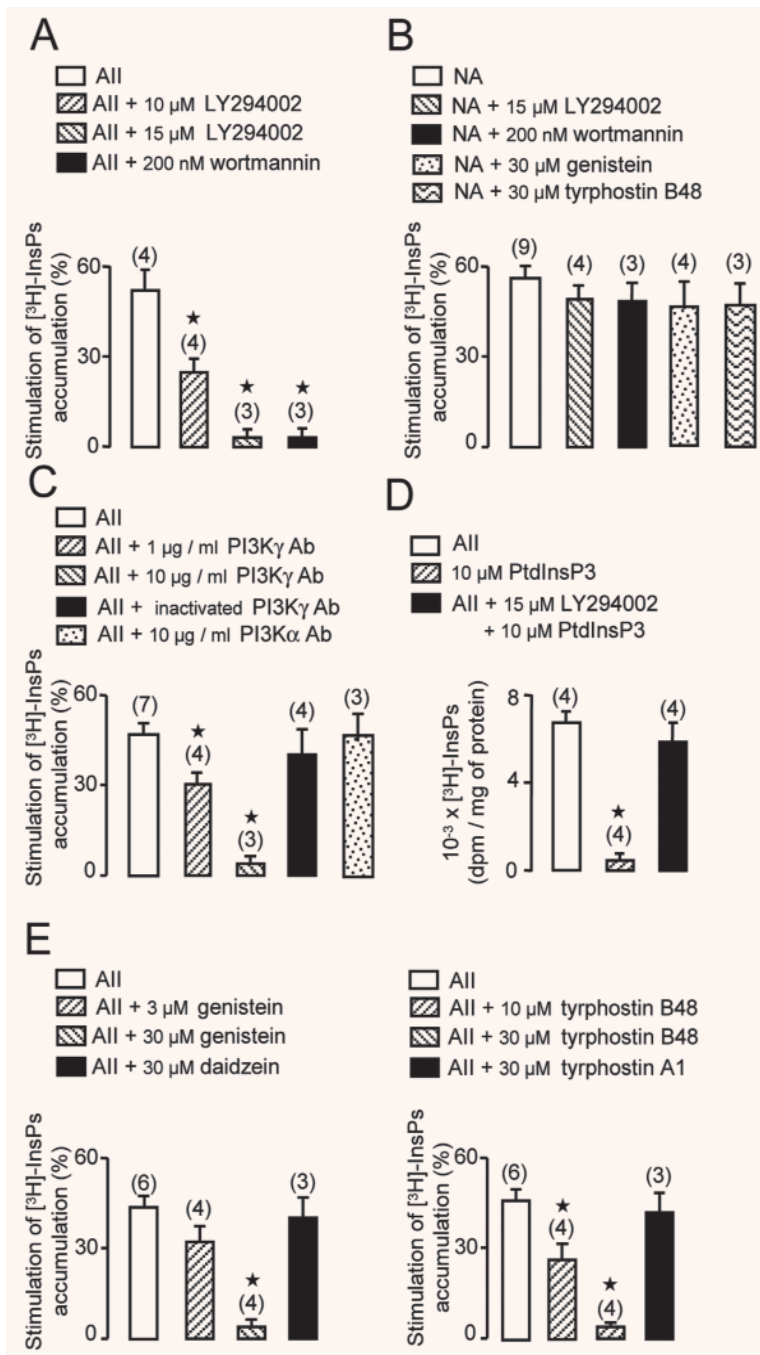


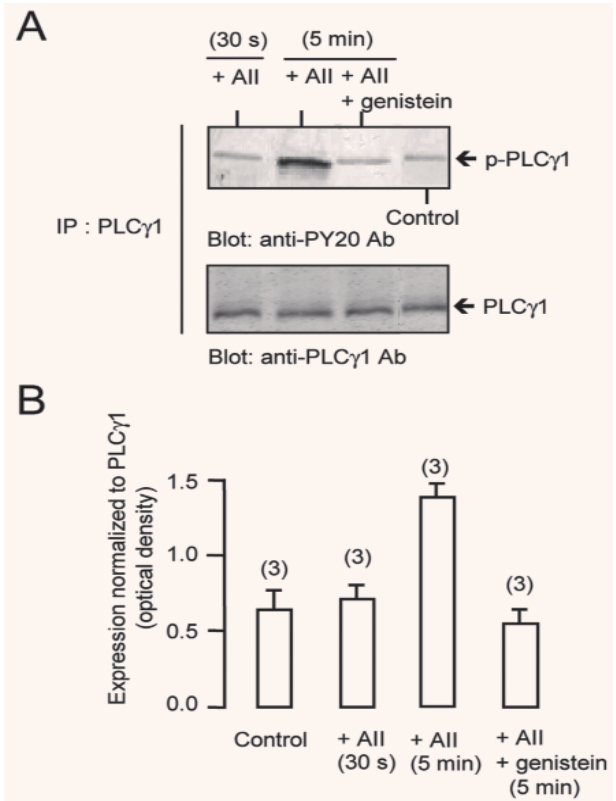
Fig. 7 Effects of inhibitors of PI3K and tyrosine kinase on AII- and NA-induced stimulation of ^3H -InsPs accumulation. **(A)** Effects of increasing concentrations of LY294002 and wortmannin on the stimulation of ^3H -InsPs accumulation induced by 10 nM AII for 5 min. Basal PLC activity was 2.9 ± 0.5 pmol InsPs / min / mg protein ($n = 4$). **(B)** Effects of LY294002, wortmannin, genistein and tyrphostin B48 on the stimulation of ^3H -InsPs accumulation induced by 10 μM NA for 10 s. Basal PLC activity was 2.8 ± 0.5 pmol InsPs / min / mg protein ($n = 6$). **(C)** Effects of increasing concentrations of anti-PI3K γ antibody, 10 $\mu\text{g/ml}$ anti-PI3K α antibody or 10 $\mu\text{g/ml}$ anti-PI3K γ antibody pre-incubated with 10 $\mu\text{g/ml}$ of its antigen peptide on the stimulation of ^3H -InsPs accumulation evoked by 10 nM AII for 5 min. Basal PLC activity was 2.6 ± 0.2 pmol InsPs / min / mg protein ($n = 5$). **(D)** Effects of 10 nM AII, 10 μM PtdInsP3 or a combination of 10 nM AII + 15 μM LY294002 + 10 μM PtdInsP3 on ^3H -InsPs accumulation (expressed as dpm/mg of protein). Basal PLC activity was 3.1 ± 0.6 pmol InsPs/min/mg protein ($n = 3$). **(E)** Effects of increasing concentrations of genistein and tyrphostine B48 and of their inactive controls, daidzein (30 μM) and tyrphostine A1 (30 μM) on the stimulation of ^3H -InsPs accumulation induced by 10 nM AII for 5 min. Basal PLC activity was 2.8 ± 0.3 pmol InsPs / min/mg protein ($n = 8$). Data are means \pm s.e. mean with the number of experiments indicated in parentheses. *, values significantly different from those obtained in control conditions ($p < 0.05$).

InsPs accumulation and NA-induced contraction. Although previous data have reported that AII may stimulate tyrosine kinase activity in vascular myocytes [6, 7, 35], we showed an increased tyrosine phosphorylation of PLC γ 1 by applications of AII for 5 min but not for 30 s; this effect was selectively inhibited by a simultaneous application of genistein. It has been reported that tyrosine phosphorylation of PLC γ 1 could be mediated by non-

receptor tyrosine kinases including Src family tyrosine kinases and JAK or EGF receptors [6, 36]. Moreover, a protein that links AT1 receptors with PLC γ 1 called G protein-coupled receptor kinase-interacting protein-1 (GIT1) has been identified as the substrate for c-Src undergoing tyrosine phosphorylation in response to AII activation [37].

However, stimulation by tyrosine kinase is not the sole way for PLC γ 1 activation since PtdInsP3

Fig. 8 Phosphorylation of PLC γ 1 by AII. (A) Immunoprecipitation with anti-PLC γ 1 antibody was performed from portal vein smooth muscle microsomes (50 μ g of protein/vial), followed by Western blot with the anti-PY20 antibody in order to monitor the phosphorylation of endogenous PLC γ 1 in control conditions or after incubation in the presence of 10 nM AII for 30s or 5 min, with and without genistein (30 μ M). The lower panel demonstrates the equivalent loading of PLC γ 1 in the microsomal lysates utilized in the immunoprecipitation reactions. (B) Tyrosine-phosphorylation of PLC γ 1 normalized to PLC γ 1 expression in the different experimental conditions described in A. Data obtained from three separate experiments.



has been described as a positive modulator of PLC γ 1 [9, 10, 38]. By using both wortmannin and LY294002 (selective PI3K inhibitors) and anti-PI3K isoform antibodies, we showed that AII-induced stimulation of InsPs production was also dependent on PI3K γ and that AII stimulated PI3K γ with a time course compatible with the delayed activation of PLC γ 1. However, incubation of portal vein permeabilized microsomes with PtdInsP3 alone failed to stimulate InsPs accumulation, supporting the idea of the concomitant involvement of tyrosine kinase. We may speculate that the delay (3–5 min) observed for PLC γ 1 activation by AII could be related to tyrosine phosphorylation of PLC γ 1, since the AII-induced activation of PI3K γ is observed within 10–30 s.

Our results from biochemical and functional experiments support the idea of a sequential activation of PI3K γ , tyrosine kinase and PLC γ 1 by AII in portal vein smooth muscle which may be involved in the slow and long-lasting contraction induced by AII. Time-dependent production of messenger molecules have been previously reported to provide a fine balanced array for smooth muscle contraction [39]. As summarized in Fig. 9, the transduction pathway activated by AII involves the G13 protein and the dimer

$\beta\gamma$ which activates PI3K γ to produce PtdInsP3. Both PtdInsP3 and tyrosine kinase are needed to activate PLC γ 1 which increases DAG and InsPs production. This pathway opposes to that described for NA [40, the present study] which involves the Gq protein and activation of PLC β 1 by α_q producing both DAG and InsPs. We may postulate that the AII-induced contractile responses evoked by AII and NA are differently modulated by these two transduction pathways. AII-induced contraction depends primarily on an early production of PtdInsP3 leading to stimulation of L-type Ca $^{2+}$ channels [11, 12, 41] and increase in [Ca $^{2+}$]_i *via* the calcium-induced calcium release mechanism by activation of ryanodine receptors; the delayed production of InsP3 prolongs calcium release by activation of InsP3 receptors. In addition, a PLC γ 1-induced stimulation of calcium entry during prolonged agonist stimulation may facilitate calcium store refilling and further InsP3-dependent calcium release [42, 43]. Similarly, transformation of InsP3 into InsP5 and InsP6 acting as second messengers [25] stimulates L-type Ca $^{2+}$ channels and Ca $^{2+}$ influx. In contrast, NA-induced contraction depends primarily on the fast activation of PLC β 1 by G α_q and the subsequent production of InsP3 inducing a fast release of Ca $^{2+}$ from the

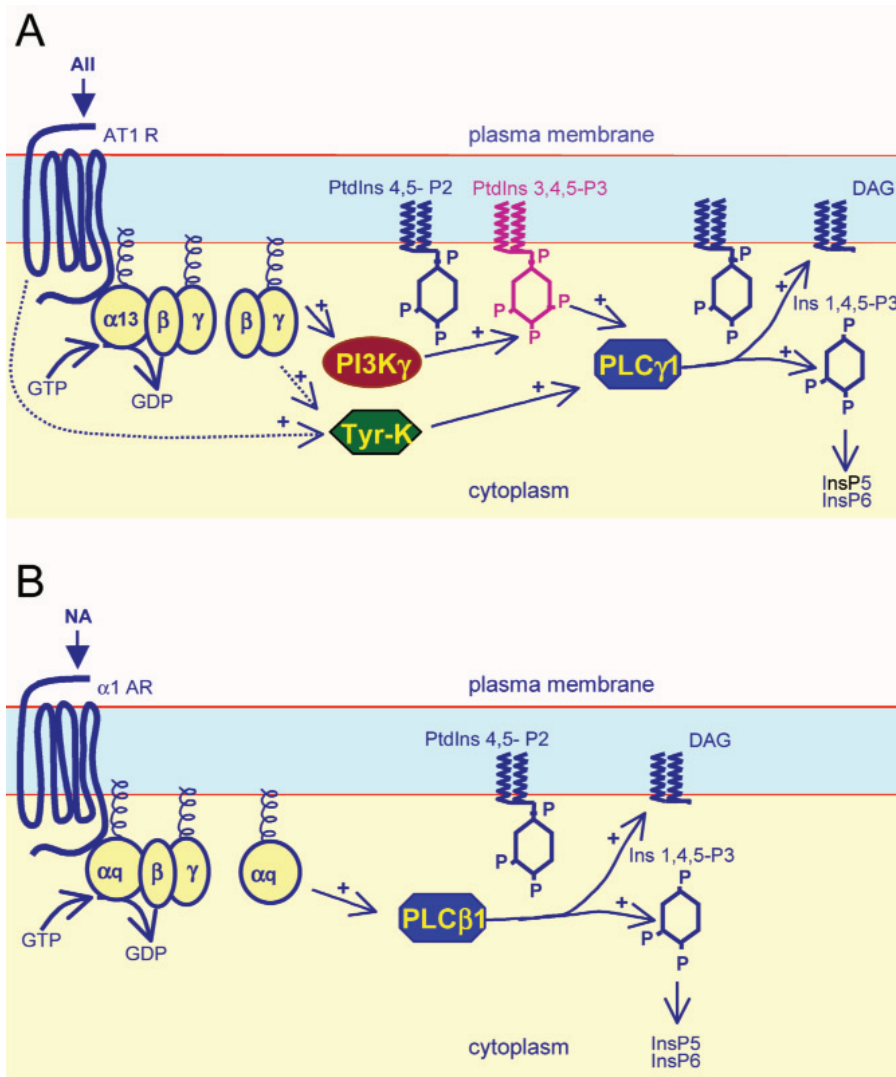


Fig. 9 Proposed transduction pathways for AII and NA in vascular myocytes. (A) Upon binding of AII to AT1 receptor, the G13 protein is dissociated and the dimer $\beta\gamma$ activates PI3K γ which phosphorylates PtdInsP2 to form PtdInsP3 [14]. PtdInsP3 has been reported to stimulate L-type Ca^{2+} channels and promote Ca^{2+} influx [41] activating calcium-induced calcium release from intracellular stores [15]. In addition, activation of PLC γ 1 needs the concomitant involvement of PtdInsP3 and tyrosine kinase. Tyrosine kinase could be activated by the AT1 receptor or alternatively by the dimer G $\beta\gamma$ (dashed lines). Consequently, there is a delayed increase in the formation of the products 1,2-diacylglycerol (DAG) which activates certain forms of protein kinase C which may stimulate L-type Ca^{2+} channels and Ins1,4,5-P3 which can mobilize the remaining Ca^{2+}

from intracellular stores. In vascular myocytes, InsP3 is rapidly transformed into InsP5 and InsP6 acting as second messengers to stimulate L-type Ca^{2+} channels [25]. (B) Binding of NA to the Gq-coupled α 1 adrenoceptor releases α q which rapidly stimulates PLC β 1 to form DAG and InsP3. In this case, the rapid and transient production of InsP3 mobilizes Ca^{2+} from intracellular stores, as previously reported [40]. This effect is also prolonged by the formation of DAG activating protein kinase C and the production of InsP5 and InsP6, both stimulating L-type Ca^{2+} channels and Ca^{2+} influx.

intracellular stores. This effect is prolonged by the production of DAG and InsP5 / InsP6 stimulating L-type Ca^{2+} channels and Ca^{2+} influx.

In conclusion, our data report that in vascular smooth muscle AII activates a complex transduction pathway involving (1) the early activation of PI3K γ by G $\beta\gamma$ and the subsequent stimulation of L-type Ca^{2+} channels and (2) the delayed stimulation of InsPs production *via* PLC γ 1 needing the concomitant participation of PI3K γ and tyrosine kinase. Both couplings participate to the increase in $[\text{Ca}^{2+}]_i$ by stimu-

lating Ca^{2+} release from the sarcoplasmic reticulum and Ca^{2+} influx through L-type Ca^{2+} channels.

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