Stimulation of two vascular smooth muscle-derived cell lines by angiotensin II: differential second messenger responses leading to mitogenesis

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1 We show here that angiotensin II (AII) and endothelin-1 (ET-1) stimulate [³H]-thymidine incorporation in a smooth muscle cell line derived from aortae of spontaneously hypertensive rats (SHR), but not in cells derived from normotensive controls (WKY). We have used the differential response of the two cell lines to investigate the relationship between second messenger systems and the mitogenic response.

2 AII produced an increase in accumulation of inositol 1,4,5-triphosphate which was greater in the SHR-derived cell line than in the WKY cells.

3 AII gave an increase in cytosolic Ca²⁺ in each of the cell lines, with both a larger peak (15–30 s) and plateau response (2 min) in the SHR cells. ET-1 gave an enhanced response in the SHR-derived cells with respect to the peak but not the plateau of cytosolic Ca²⁺.

4 Phospholipase D activity was studied by monitoring the formation of [³P]-phosphatidylbutanol in ³²Pi prelabelled cells. AII stimulation gave a larger phospholipase D response in SHR-derived cells, while ET-1 gave a larger response in WKY-derived cells.

5 Stimulation of SHR-derived cells with 100 nM AII for 1 h, followed by 19 h in the absence of agonist, stimulated [³H]-thymidine incorporation over the next 4 h. When the 1 h stimulation with AII was in the presence of increasing concentrations of butanol, which diverts the product of the phospholipase D pathway, there was a loss of stimulated [³H]-thymidine incorporation which was significant at 10 mM butanol and at 30–50 mM reached a maximum loss of 40%.

6 Contrasting with this there was no apparent loss of ET-1-stimulated thymidine incorporation when butanol was present at concentrations up to 40 mM.

7 These results suggest that phospholipase D is one of several pathways in the mitogenic response of SHR-derived vascular smooth muscle cells to AII.

Keywords: Angiotensin II; endothelin-1; vascular smooth muscle; mitogenesis; phospholipase D; phospholipase C

Introduction

Cell surface receptors known to control the cell cycle fall into two principal groups; those with a single transmembrane span and intrinsic tyrosine kinase activity, and those with seven transmembrane domains which are linked to diverse effector mechanisms by heterotrimeric G-proteins (e.g. Pouyssegur & Seuwen, 1992). Recently there has been considerable progress in understanding how the former class, on activation by growth factors such as platelet-derived growth factor, initiates a sequence of events leading to mitogenesis. The mechanisms linking the seven transmembrane receptors to cellular proliferation are more obscure, but often involve a (poly)phosphoinositide specific phospholipase C (PLC) as the primary effector mechanism linked to the receptor by a heterotrimeric G protein. This signalling pathway generates elevated intracellular Ca²⁺ and activation of protein kinase C. While both these have been implicated in mitogenic signalling by seven transmembrane receptors, it is now apparent that there is convergence in the mechanisms underlying cell cycle control between the two principal types of receptors involved. One component which has received some interest recently is the activation of phospholipase D (PLD). This enzyme cleaves specific phospholipids to produce phosphatidic acid as the lipid fragment. It has been shown that in certain cases, activation of tyrosine kinase by growth factors leads to stimulation of PLD (e.g. Zhang *et al.*, 1992; Fukami & Takenawa, 1992; Cook & Wakelam, 1992). There are

numerous reports of activation of PLD by receptors which are linked to PLC; in some of these cases tyrosine kinases have also been implicated in the link from receptor to PLD (Uings *et al.*, 1992; Wilkes *et al.*, 1993). Activation of PLD may therefore be considered as an example of convergence of signalling pathways activated by the two classes of receptor.

Evidence for considering the involvement of PLD in certain instances of mitogenic signalling has recently been summarised (Boarder, 1994). The case for a role for PLD is particularly strong for mitogenic responses in vascular smooth muscle cells. In the present study we have utilised two rat aorta-derived cell lines which have different mitogenic responses to both angiotensin II (AII) and endothelin-1 (ET-1) to investigate the relationship between the mitogenic response and the stimulation of PLC and PLD. The two cell lines are derived from one strain of Wistar rat, SHR, which spontaneously develops hypertension and another, WKY, which remains normotensive. It has previously been reported that the SHR-derived cells show an enhanced response to AII and ET-1 with respect to mitogenesis (Paquet *et al.*, 1990; Bunkenberg *et al.*, 1992) and PLC (Resink *et al.*, 1989; Paquet *et al.*, 1990; Osani & Dunn, 1992) but that there were no differences in receptor numbers and affinities between the two cell types (Bolger *et al.*, 1991; Osani & Dunn, 1992). A recent report has shown that SHR-derived cells have an enhanced PLD response to platelet-derived growth factor, which activates an intrinsic tyrosine kinase receptor (Kondo *et al.*, 1994). We have used these differences between these

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cell lines to investigate the relationship between the PLC signalling cascade, PLD activation, and the mitogenic response to activation of seven transmembrane receptors. The experiments described provide support for a role for PLD in the mitogenic response to G-protein:PLC linked receptors in cultured vascular smooth muscle cells, but indicate that this relationship is not a simple one.

Methods

Cell preparation and culture

Cells were prepared by the method described by Davies *et al.* (1991). After determining the arterial pressure of 12 week-old SHR and WKY rats (supplied from colonies maintained by the Biomedical Services Unit of Leicester University) by use of a tail cuff, the animals were decapitated and the thoracic aorta removed and stripped of adventitia. The media was cut into 1 mm pieces and enzymatically digested. Following centrifugation the cells were resuspended in growth medium. Clonal cultures were established and after 5 days those colonies with smooth muscle cell morphology were combined. Growth medium comprised Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, penicillin (100 i.u. ml^{-1}), streptomycin ($100 \mu\text{g ml}^{-1}$) and glutamine (27 mg ml^{-1}). Cells were routinely cultured in 50 ml of the above medium in a 175 cm^2 flask at 37°C in 5% CO_2 , 95% air. Cells were used for experiments between passages 6 and 12, seeded into 24 well plates. Cells showed 100% positive smooth muscle actin immunofluorescence.

Incorporation of [^3H]-thymidine

Cells at 80% confluence were maintained serum-free for 24 h and then exposed to agonist at the concentration indicated, in a serum-free medium, for 20 h. [^3H]-thymidine ($0.074 \text{ MBq ml}^{-1}$) was then added followed by a further 4 h incubation. The medium was then aspirated and the cells washed twice with balanced salt solution (BSS, mM: NaCl 125, KCl 5.4, NaHCO_3 16.2, HEPES 30, NaH_2PO_4 1, MgSO_4 0.8, CaCl_2 1.8, glucose 5.5; buffered to pH 7.4 with NaOH and gassed with 95% O_2 5% CO_2). The cell monolayer was then placed on ice and washed sequentially with ice cold 5% trichloroacetic acid and ethanol, taken up into 0.1 M NaOH and scintillation counted. An alternative protocol designed for the butanol experiments employed a 1 h stimulation with the agonist followed by a 19 h incubation with no agonist in serum-free medium before incubation with [^3H]-thymidine and extraction as before. When butanol was present it was added 5 min before the agonist and during incubation with agonist, but it was not present during the subsequent periods.

Measurement of $\text{Ins}(1,4,5)\text{P}_3$ levels

Cells in 24 well multiwells were washed twice with 1 ml BSS. Agonists were then added in $200 \mu\text{l}$ of BSS. The incubation was stopped by addition of $100 \mu\text{l}$ cold 1.5 M trichloroacetic acid. The acid extract was washed with diethylether and assayed for mass of $\text{Ins}(1,4,5)\text{P}_3$ by the protein binding procedure of Challiss *et al.* (1988).

Measurement of free intracellular Ca^{2+}

Cells were grown to just reach a confluent monolayer on $9 \times 22 \text{ mm}$ glass coverslips and then cultured serum-free for 24 h. The cells were loaded for 1 h at 37°C with $4 \mu\text{M}$ Fura-2 acetoxymethyl ester in Medium 199 buffered to pH 7.4 and supplemented with 10 mM HEPES and 0.1% bovine serum albumin (BSA), followed by 30 min in the absence of the dye. Fluorescence was measured with the coverslip inserted diagonally in a quartz cuvette in 1.8 mM CaCl_2 , 0.8 mM

MgSO_4 , 15 mM HEPES, 140 mM NaCl, 5 mM KCl and 5 mM glucose, pH 7.4. A Photon Technology International Delta-scan dual excitation fluorimeter was used with excitation wavelengths of 340 and 380 nm (5 readings per second) and an emission wavelength of 530 nm, according to the principle described by Grynkiewicz *et al.* (1985). Data are presented as the ratio of the dual wavelength readings.

Assay for phospholipase D activity

PLD activity in intact cells was measured as described in Purkiss & Boarder (1992) and Boarder & Purkiss (1993). Briefly, cells just at confluence in 24 wells were labelled for 24 h at 37°C in a phosphate-free BSS with [^{32}P]-orthophosphoric acid (0.25 MBq ml^{-1}). Preincubations for 10 min included butanol added to the labelling medium at a final concentration of 50 mM, following which the medium was removed and the cells stimulated in BSS with 50 mM butanol and agonists as required until this incubation period was stopped by addition of cold methanol. Drugs were added as indicated to both the preincubation and the incubations. Following extraction with chloroform the [^{32}P]-labelled phosphatidylbutanol and phosphatidic acid were separated from other phospholipids by thin layer chromatography on oxalate coated silica plates developed in ethyl acetate:acetic acid:2,2,4-trimethylpentane (9:2:5). The location of phosphatidic acid and phosphatidylbutanol was established by autoradiography and comparison with iodine stained standards, and the spots scraped and scintillation counted. We have previously discussed evidence that this procedure converts essentially all the PLD products to phosphatidylbutanol (Purkiss & Boarder, 1992; Boarder & Purkiss, 1993), in which case the agonist stimulated counts in the phosphatidylbutanol spot are an index of PLD activity.

Materials

[^3H]-thymidine, [^{32}P]- P_i and [^3H]- $\text{Ins}(1,4,5)\text{P}_3$ were purchased from Amersham (Buckinghamshire, UK). Angiotensin II (AII), insulin and fura-2-acetoxymethyl ester were obtained from Sigma (Dorset, UK) and endothelin-1 (ET-1) was obtained from the Peptide Research Institute (Osaka, Japan).

Results

[^3H]-thymidine incorporation in response to ET-1 and AII

Initial experiments set out to characterize the stimulation of [^3H]-thymidine incorporation into both SHR and WKY cells by various agonists. Figure 1 shows the results of stimulation with ET-1, AII and insulin. The results with WKY cells show that insulin ($1 \mu\text{M}$), but neither ET-1 nor AII at 100 nM, were capable of stimulating thymidine incorporation (Figure 1a). Insulin combined with either of the other two agonists produced a response which was sometimes, but not reliably, greater than with insulin alone. By contrast the SHR cells gave no response to insulin but a substantial response to both AII and ET-1 (Figure 1b). This dissociation of responses indicated that the two cell types differed in their control of mitogenesis, leading to the subsequent studies. A series of experiments examining the concentration-response relationship for AII and ET-1 stimulation of SHR cells is illustrated in Figure 2, indicating EC_{50} values at low nM levels for both agonists.

Phospholipase C responses to AII and ET-1

The action of AII and ET-1 found on vascular smooth muscle cells are predominantly at AT_1 and ET_A receptors respectively, both of which are G-protein linked to PLC

Figure 1 Incorporation of [³H]-thymidine into (a), WKY-derived vascular smooth muscle cells and (b) SHR-derived cells. Agonists were present (angiotensin II AII, and endothelin-1 ET-1 at 100 nM and insulin at 1 μ M) as indicated for 24 h, during the last 4 h of which [³H]-thymidine was also present. The results are expressed as counts present in DNA extracted from a well of a 24 well plate, and are mean $\pm$ s.e. mean ($n = 3$) from a single experiment representative of 3 separate experiments.

(Griendling *et al.*, 1986; Peach & Dostal, 1990; Sakurai *et al.*, 1992) so we investigated the accumulation of Ins(1,4,5)P₃ in response to these two agonists. Figure 3a shows the time course of Ins(1,4,5)P₃ accumulation in SHR and WKY cells to stimulation with 100 nM AII. Starting from the same unstimulated levels (Figure 3a), the peak was at 15 s with both cell types, but higher stimulated levels of Ins(1,4,5)P₃ were seen in SHR than WKY cells. Over 4 separate experiments the peak (15 s) response of the SHR cells was 3.00 ± 0.44 ($P < 0.01$) fold that of the WKY cells, while the plateau at 2 min was 2.36 ± 1.38 fold (NS), reflecting variation in the presence of a plateau in WKY cells (data are arithmetic mean $\pm$ s.e. mean, $n = 4$, in each case). Using 15 s stimulation the concentration-response relationship for AII was investigated. It was characteristic of these experiments that the higher concentrations of AII gave a reduced response. Figure 3b shows the results of both cell types. Pooled across 4 experiments the apparent EC₅₀ values (taken as the concentration of agonist giving 50% of the peak response on the rising phase of the curve) were 50.9 ± 10.0 nM for SHR cells and 46.7 ± 7.6 nM for WKY cells ($n = 4$). Investigation of the time course of raised Ins(1,4,5)P₃ at these concentrations suggested that this reduced response at higher concentrations was not due to a change in the time of the peak response (data not shown).

A similar series of experiments was undertaken with ET-1 (data not shown). The peak response to ET-1 was at 30 s for both cell types, but the height of this peak for SHR cells was 4.56 ± 0.72 fold that of the WKY cells ($P < 0.05$) while the plateau at 2 min was 3.33 ± 1.93 fold (NS).

Figure 2 Concentration-response relationship for [³H]-thymidine incorporation into SHR-derived vascular smooth muscle cells with increasing concentrations of (a) angiotensin II (AII) and (b) endothelin-1 (ET-1). Data are normalised to % maximum response and are pooled across 3 separate experiments; error bars are s.e. mean.

Ca²⁺ responses to AII and ET-1

PLC-linked receptors regulate intracellular Ca²⁺ by Ins(1,4,5)P₃-mediated mobilization of intracellular Ca²⁺ pools and stimulation of Ca²⁺ entry. The change in Ca²⁺ may be related to mitogenic signalling, so we characterized this response in the two cell types. Figure 4 shows typical traces. The responses to ET-1 (100 nM) and to AII (100 nM) were of a similar magnitude. Data not shown established that the plateau phase of these responses, but not the majority of the initial peak, was dependent on extracellular Ca²⁺. In both cases the magnitude of peak responses was higher in the SHR than the WKY cells. For AII the peak response (expressed as fluorimetric ratios) was 0.865 ± 0.135 in the SHR cells and 0.445 ± 0.067 in the WKY cells ($n = 7$, $P < 0.05$), while the plateau was 0.568 ± 0.029 in the SHR cells and 0.169 ± 0.085 in the WKY cells ($n = 7$, $P < 0.001$). For ET-1 the peak response was 0.929 ± 0.089 for the SHR cells and 0.526 ± 0.036 for the WKY cells ($n = 3$, $P < 0.05$), while the plateau was 0.549 ± 0.061 for the SHR cells and 0.429 ± 0.020 for the WKY cells (NS).

PLD responses to AII and ET-1

To establish the degree of stimulation of PLD in these cells the formation of [³H]-phosphatidylbutanol, a unique product of the PLD pathway, was measured. The time course of [³²P]-phosphatidylbutanol accumulation following stimulation of SHR and WKY cells by 100 nM AII is illustrated in Figure 5a, while Figure 5b shows [³²P]-phosphatidylbutanol

Figure 3 (a) Time course of inositol (1,4,5)trisphosphate (Ins(1,4,5)P₃) accumulation in WKY cells (■) and SHR cells (□) in response to 100 nM angiotensin II (AII). (b) Concentration-response curves for accumulation of Ins(1,4,5)P₃ with 15 s stimulation by AII in WKY cells (■) and SHR (□). Data are in each case from a single experiment (mean $\pm$ s.e. mean, $n = 3$) representative of 4 separate experiments; data pooled across experiments are given in the text.

Figure 4 Stimulation of cytosolic Ca²⁺ in WKY- and SHR-derived cells in response to 100 nM angiotensin II (AII) (a) and endothelin-1 (ET-1) (b). Calcium was measured by the Fura 2 procedure and increases indicated by elevations in the ratio fluorescence at excitation wavelengths of 340 and 380 nm. The traces shown are representative of 3 for ET-1 and 7 for AII. Data collected across experiments are presented in the text.

accumulation in response to increasing concentrations of AII. In each case the stimulation of PLD by AII showed a similar concentration-response relationship. At the highest concentration of AII used there was a modestly larger response in the SHR cells. Pooled across 3 separate experiments, the response of the WKY cells to AII at 1 μ M was $64 \pm 8\%$ that of the response of the SHR cells (in each case calculated as c.p.m. [³²P]-phosphatidylbutanol mg⁻¹ of protein).

Similar experiments were carried out with ET-1. Figure 6 shows the time course of [³²P]-phosphatidylbutanol accumulation in the 2 cell types in response to a maximally effective (100 nM) concentration of ET-1. Here the results were very different from those with AII stimulation. When SHR cells were stimulated with ET-1 there was little if any increase in [³²P]-phosphatidylbutanol accumulation, while when WKY cells were stimulated there was a large and sustained increase in the levels of this PLD product. This indicates that in response to ET-1 the WKY cells, but not the SHR cells, exhibited a substantial increase in PLD activity.

Effect of butanol on AII-stimulated [³H]-thymidine incorporation

In preliminary experiments we found that we were able to stimulate [³H]-thymidine incorporation in cells just reaching confluence by the following protocol: 24 h serum-free medium prior to and during the experiment, 1 h AII (100 nM), 19 h serum-free medium, 4 h serum-free medium with [³H]-thymidine (0.5 μ Ci ml⁻¹). This was followed by the standard extraction procedure described. The presence of AII

for 1 h gave a stimulation of [³H]-thymidine incorporation 20–24 h later which was several fold over basal. We used this protocol to test for the effect of butanol during the incubation period in the presence of AII. Butanol will inhibit the formation of phosphatidic acid by the PLD pathway in a concentration-dependent manner. The relatively short stimulation with agonist enables the experiment to be carried out without the necessity for prolonged exposure of cells to high concentrations of butanol. A preincubation with butanol for 5 min preceded the stimulation period, so cells were exposed to butanol for 65 min. Results shown in Figure 7a indicate that butanol partially inhibits the AII-stimulated incorporation of [³H]-thymidine. The effect of exposure to butanol was apparent with the lowest concentration used (10 mM). The effect of butanol at concentrations of 30 to 50 mM was similar (Figure 7a). At 50 mM, butanol inhibited $40 \pm 6\%$ ($n = 4$ separate experiments, each in triplicate) of the AII-stimulated [³H]-thymidine incorporation. In contrast, there was little effect of butanol at concentrations up to 40 mM on ET-1-stimulated [³H]-thymidine incorporation in SHR cells (Figure 7b).

Effect of chromofus PLD

In a previous report on vascular smooth muscle cells it was shown that addition of an exogenous PLD preparation led to enhanced [³H]-thymidine incorporation (Kondo *et al.*, 1992). Here, WKY and SHR cells were incubated with increasing concentrations of *Streptomyces chromofus* PLD (0.5–3 μ g ml⁻¹) for 24 h with the addition of [³H]-thymidine

Figure 5 Phospholipase D (PLD) stimulation in WKY- (●) and SHR- (○) derived cells in response to (a), incubation for increasing time with 100 nM angiotensin II (AII); (b) incubation with increasing concentrations of AII for 5 min. PLD is expressed as the agonist stimulated accumulation of $[^{32}\text{P}]\text{-phosphatidylbutanol}$ in the presence of 50 mM butanol. Data are expressed as fold over basal and pooled across 3 experiments; with s.e. mean shown. Two way analysis of variance of data in (b) showed a significant difference between the two cell types ($P < 0.001$) in response to the agonist; there was no difference between the cells in the unstimulated levels.

Figure 6 Time course of phospholipase D (PLD) stimulation in WKY- (■) and SHR- (□)-derived cells in response to endothelin-1 (ET-1, 100 nM). Data are basal subtracted and pooled across 3 experiments; s.e. mean are shown.

for the final 4 h, as described for the final procedure. A small increase in incorporation was apparent in some instances; however no consistent stimulation was seen (data not shown).

Figure 7 Stimulation of $[^3\text{H}]\text{-thymidine}$ incorporation into SHR-derived vascular smooth muscle cells by (a) 100 nM angiotensin II (AII) and (b) 100 nM endothelin-1 (ET-1) in the presence of increasing concentrations of butanol. The cells were preincubated with butanol where appropriate for 5 min, incubated with butanol and the agonist for 60 min, incubated with neither butanol nor agonist for 19 h, followed by a 4 h incubation in the presence of $[^3\text{H}]\text{-thymidine}$ and extraction of labelled DNA. The experiment is one of three with closely similar results (pooled data in text). *Significantly different ($P < 0.005$ or greater) from stimulation in the absence of butanol (Student's unpaired *t* test).

Discussion

The observation described here that ET-1 and AII stimulate $[^3\text{H}]\text{-thymidine}$ incorporation in SHR, but not WKY, cells is consistent with earlier reports of enhanced mitogenicity of the SHR-derived cells (Paquet *et al.*, 1990; Bunkenberg *et al.*, 1990). The observation in our two cell lines that $[^3\text{H}]\text{-thymidine}$ responses to insulin show the converse relationship (i.e. greater responses in WKY- than SHR-derived cells) was unexpected, and a reflection of the complexity of the signalling pathways that control mitogenesis.

Several papers have documented enhanced PLC responses in SHR-derived smooth muscle cells (Resink *et al.*, 1989; Paquet *et al.*, 1990; Osani & Dunn, 1992). This is the first report characterizing the accumulation of $\text{Ins}(1,4,5)\text{P}_3$ in response to stimulation with agonists in SHR- and WKY-derived cells. This is important because this is the inositol phosphate isomer produced by the action of PLC on phosphatidylinositol trisphosphate and because it is the level of this isomer which determines the Ca^{2+} response. The basal levels of both $\text{Ins}(1,4,5)\text{P}_3$ and Ca^{2+} were the same for the two cell types. However both $\text{Ins}(1,4,5)\text{P}_3$ and Ca^{2+} were the same for the two cell types. However both $\text{Ins}(1,4,5)\text{P}_3$ and Ca^{2+} plateau responses to the two agonists were higher for the SHR than the WKY cells, with the exception of the plateau Ca^{2+} response to ET-1. The elevated Ca^{2+} respon-

siveness of SHR-derived cells has been described in a number of studies (e.g. Nabika *et al.*, 1985; Sugiyama *et al.*, 1986), although there are contrary reports of no difference between the cell types (Neusser *et al.*, 1993).

The mechanism by which G-protein-linked receptors activate PLC in vascular smooth muscle cells is unclear. This is because while PLC γ and PLC δ have been reported in these cells, there is an apparent absence of PLC β (Kato *et al.*, 1992; Homma *et al.*, 1993; Morrero *et al.*, 1994). It is the PLC β isoforms which are activated by dissociated G-protein subunits, whether they are α -GTP or $\beta\gamma$ subunits. It has been shown that SHR and WKY cells differ with respect to PLC δ (Kato *et al.*, 1992). This enzyme has no confirmed mode of activation; it is not stimulated by G-protein α -subunits, and stimulation by $\beta\gamma$ -subunits is weak compared to that of PLC β isoforms (Park *et al.*, 1993). More significant is its sensitivity to elevations in cytosolic Ca²⁺; it is characteristic of the SHR form of the enzyme that it shows an enhanced responsiveness to modest rises in cytosolic Ca²⁺. Separate studies have shown that in vascular smooth muscle cells, activation by AII or ET-1 of PLC or PLD activity is sensitive to tyrosine kinase inhibition (Wilkes *et al.*, 1993; Morrero *et al.*, 1994); it has also been shown that stimulation by AII and ET-1 leads to tyrosine phosphorylations and activation of mitogen activated protein (MAP) kinases (Ishida *et al.*, 1992; Koide *et al.*, 1992; Molloy *et al.*, 1993) and PLC γ 1 (Morrero *et al.*, 1994). With respect to the differential PLC response of the two cell types, it is likely that this is due to the difference in PLC δ response, possibly downstream of a priming activation of PLC γ or undetectable, but functional, amounts of PLC β .

Various mechanisms have been proposed whereby heterotrimeric G-protein-linked receptors activate PLD, including direct G-protein links and downstream of raised cytosolic Ca²⁺ and activation of protein kinase C (see Boarder, 1994, for summary). PLD is also activated by growth factors acting on intrinsic tyrosine kinase-linked receptors (Zhang *et al.*, 1992), in a manner which may be unrelated to PLC activation (Fukami & Takenawa, 1992; Cook & Wakelam, 1992). Recent elegant studies have shown that PLD may be activated downstream of a small G-protein called ADP-ribosylating factor, (Brown *et al.*, 1993; Cockcroft *et al.*, 1993). In vascular smooth muscle cells we have shown that tyrosine kinase activity is required for the stimulation of PLD by ET-1 (Wilkes *et al.*, 1993); this has also been confirmed for AII stimulation of mitogenesis in the cells used in the present study (C. Morton and M.R. Boarder, unpublished observations). The observation that in these cell lines the PLD response to ET-1 was higher in the WKY cells was contrary to our expectations. In this case the mitogenic and PLC responses were not correlated with the PLD response, making any simple causal relationship between them difficult to support. However, in the case of the AII stimulation the three responses, mitogenic, PLC and PLD, were each higher in the SHR than the WKY cells.

The lack of correlation between the PLD responses to ET-1 and the mitogenic response does not exclude an involvement of PLD in the mitogenic response when AII was the agonist. To investigate these possibilities further we stimulated with AII and examined the consequence of co-incubation of the agonist with butanol, to divert the product of PLD activation through the phosphatidylol reaction to phosphatidylbutanol. Previously we have provided evidence that 40 mM butanol can convert essentially all the PLD product away from phosphatidic acid formation while leaving the PLC response intact (Purkiss & Boarder, 1993; Boarder & Purkiss, 1993). In the absence of a suitable PLD inhibitor, butanol provides a method of inhibiting phosphatidic acid formation by PLD. To avoid the necessity of incubating cells with high butanol concentrations for many hours, we used a relatively short incubation with agonist

followed by a long lag period before measuring thymidine incorporation. It had previously been shown that brief stimulations with either exogenous PLD or with PLD activating concentrations of platelet-derived growth factor were able to produce a mitogenic response in vascular smooth muscle cells many hours later (Kondo *et al.*, 1992). Here we have shown that 1 h stimulation with AII and measurement of [³H]-thymidine incorporation after a lag of 19 h is as effective as continuous 20 h stimulation with AII. The mitogenic signalling process initiated during this 1 h period is in part dependent on PLD, as indicated by the inhibition of mitogenesis by butanol at the same concentration range as the diversion of the PLD reaction. However, the dependency was only partial, since the effect of butanol formed a plateau between 30 and 40 mM, leaving about 60% of the mitogenic response intact.

These results provide support for a role for PLD in the mitogenic response of SHR-derived vascular smooth muscle cells to stimulation with AII. While other effects of high butanol concentrations cannot be excluded, we have shown that other signalling pathways remain intact (Boarder & Purkiss, 1993). This includes the demonstration that the stimulation of PLC by ET-1 in rat aorta-derived smooth muscle cells was unaffected by 24 h preincubation with 50 mM butanol, and that after 24 h preincubation with 50 mM butanol, stimulation with insulin was still able to elicit a mitogenic response in A10 cells (Wilkes *et al.*, 1993). Here we have used a protocol which reduces the time of exposure to butanol, and have shown that the plateau of butanol inhibition occurs at the concentrations expected for diversion of the PLD pathway; the observation that this leaves a substantial part of the mitogenic response intact may itself be taken as an indication that the butanol effect on thymidine incorporation is not non-specific.

This conclusion is confirmed by the resistance of ET-1-stimulated thymidine incorporation to the inhibitory influence of butanol up to a concentration of 40 mM. Combined with the lower ET-1 stimulated PLD in SHR compared to WKY cells, these results show that PLD stimulation is not significant in the mitogenic response to ET-1 in the SHR-derived cells. The results point to a difference in the mitogenic signalling mechanisms associated with AT₁ and ET_A receptors in this cell line.

The failure of exogenous PLD to produce a reliable mitogenic response is surprising in view of an earlier report (Kondo *et al.*, 1992), and may be taken as an indication that elevated PLD, while necessary for the full response to AII, is not sufficient in itself to elicit enhanced DNA synthesis. However, there are some inherent complications in understanding the consequences of adding phospholipase preparations to the outside of cells, such as the ability to stimulate activity which is effective inside the cells, and non-specific lipid damage. These complications make this experiment difficult to interpret.

The concentration-response data obtained here show that the stimulation of thymidine incorporation lies to the left of the PLC and PLD responses. This is presumably a reflection of multiple steps downstream of the phospholipases, and possibly of involvement of separate pathways controlling mitogenesis by these receptors in these cells.

The results obtained here also suggest that part of the mitogenic response to heterotrimeric G-protein linked receptors is not dependent on PLD. This view is suggested by the results with ET-1 stimulation, and is reinforced by the partial nature of the inhibition of thymidine incorporation by butanol in response to AII. Further studies are underway to investigate a possible role of the Ras:MAP kinase cascade and tyrosine phosphorylations in the mitogenic response to seven transmembrane receptors in vascular smooth muscle cells.

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