Stimulated Mitogen-activated Protein Kinase Is Necessary but Not Sufficient for the Mitogenic Response to Angiotensin II

A ROLE FOR PHOSPHOLIPASE D*

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Activation of the mitogen-activated protein kinase (MAPK) cascade has been widely associated with cell proliferation; previous studies have shown that angiotensin II (AII), acting on 7-transmembrane G protein-coupled receptors, stimulates the MAPK pathway. In this report we investigate whether the MAPK pathway is required for the mitogenic response to AII stimulation of vascular smooth muscle cells derived from the hypertensive rat (SHR-VSM). AII stimulates the phosphorylation of MAPK, as determined by Western blot specific for the tyrosine 204 phosphorylated form of the protein. This MAPK phosphorylation was inhibited by the presence of the inhibitor of MAPK kinase activation, PD 098059. Using a peptide kinase assay shown to measure the p42 and p44 isoforms of MAPK, the stimulated response to AII was inhibited by PD 098059 with an IC₅₀ of 15.6 ± 1.6 μM. The AII stimulation of [³H]thymidine incorporation was inhibited by PD 098059 with an IC₅₀ of 17.8 ± 3.1 μM. PD 098059 had no effect on AII-stimulated phospholipase C or phospholipase D (PLD) activity. When the SHR-VSM cells were stimulated with phorbol ester, there was an activation of MAPK similar in size and duration to the response to AII, but there was no significant enhancement of [³H]thymidine incorporation. There was also no activation of PLD by phorbol ester, while AII produced a robust PLD response. Diversification of the product of the PLD reaction by 1-butanol caused a partial loss of the [³H]thymidine response; this did not occur with tertiary butanol, which did not interfere with the PLD reaction. These results show that in these cells the MAPK cascade is required but not sufficient for the mitogenic response to AII, and suggest that the full mitogenic response requires both MAPK in conjunction with other signaling components, one of which is PLD.

Angiotensin II (AII) acts at two G protein-coupled 7-transmembrane receptors, AT₁ (1) and AT₂ (2), in its role as a major regulator of cardiovascular function. AII has been shown to be a growth factor (3), either alone or as a co-mitogen, in diverse cell types. In the cardiovascular system this is considered of importance due to the contribution of vascular smooth muscle proliferation in the development of atherosclerotic plaques and thickening of the blood vessel wall in hypertension, and due to the contribution of AII-stimulated myocyte and fibroblast proliferation in the development of cardiac hypertrophy. In vascular and cardiac tissue remodeling both hypertrophy and hyperplasia are important responses to AII. For example, AII acts at AT₁ receptors inducing hyperplasia of cultured cardiac fibroblasts, but hypertrophy without hyperplasia of cultured cardiac myocytes (4, 5). In cultured rat aortic smooth muscle cells (RASM), AII stimulation of AT₁ receptors has been shown to elicit a mitogenic response (6). In several studies it has been shown that AII induces proliferation only in conjunction with other growth factors, when it acts as a progression factor, promoting but not initiating cellular division (7, 8). However, it has been shown in several reports that AII acts as a complete mitogen in RASM cultures from spontaneously hypertensive rats (SHR), while giving little or no response in cultures derived from normotensive controls (9–11). This difference in DNA synthesis and mitogenic response has been shown to correlate with increased phospholipase C (PLC) and phospholipase D (PLD) responses, and decreased cyclic GMP influence, in SHR-derived cells (9, 11–13).

Many components of the pathway from the AT₁ receptor to the mitogenic response have been investigated (see Ref. 14, for review). It is now widely recognized that the tyrosine kinase/mitogen-activated protein kinase (MAPK) cascades, initially investigated with respect to tyrosine kinase growth factor receptors, are widely involved in the signal transduction of heterotrimeric G protein-coupled receptors, and that multiple pathways from these receptors to the MAPK pathway exist (15). In RASM cells it is known that AII stimulation leads to MAPK activation (7, 16), and it has recently been shown that this involves sequential activation of the pp60src tyrosine kinase (17), p21ras-GTP, and Ras-Raf-1 complex formation (18). However, direct investigation of the extent to which mitogenic responses to AII are dependent on the Ras/MAPK cascade has been lacking. Evidence has been presented that it is the size of a MAPK activation (7), or the duration of the activation and extent of translocation to the nucleus (19), which determines whether there is a consequent mitogenic response. Here we show that stimuli producing the same size and time course of MAPK stimulation vary in their ability to elicit an increase in DNA synthesis in SHR-derived RASM cultures. However, selective attenuation of MAPK activation by an inhibitor of MAPK kinase (MEK) activation (PD098059; Ref. 20) abolishes the mitogenic response to AII. This suggests that MAPK activation, while required for the mitogenic response to AII, must coincide with one or more other signaling events, and we pro-

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‡ The abbreviations used are: AII, angiotensin II; RASM, cultured rat aortic smooth muscle cells; SHR, spontaneously hypertensive rats; PLC, phospholipase C; PLD, phospholipase D; MAPK, mitogen-activated protein kinases; MEK, MAPK/extracellular signal related kinase; PMA, phorbol myristate acetate.
vide some evidence that PLD (11, 21) may be one of the other contributing components in this pathway to mitogenesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture medium was purchased from Life Technologies, Inc., Paisley, Scotland, except for fetal calf serum, which was from Advance Protein Products Ltd., West Midlands, United Kingdom. The nonapeptide substrate for the MAPK assay was synthesized by the peptide synthesis laboratory at Leicester University. [γ-32P]ATP and the horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham (Bucks, UK). The antibody specific for phosphorylated MAPK was raised in rabbits against a peptide sequence corresponding to residues 196–209 of human p44 MAPK in which the tyrosine at residue 204 was phosphorylated and was purchased from New England BioProducts Ltd. (Herts, UK). [3H]Thymidine, [γ-32P]ATP, and ECL reagents for developing Western blots were from Amersham. Ro 31-8220 was a generous gift from Roche Products Ltd. (Welwyn, UK), and PD098059 was kindly donated by Dr. A. Salteia, Park Davis, Ann Arbor, MI.

**Cell Culture**—Cells were prepared, as described previously (22), using thoracic aorta from 12-week-old SHR rats (from colonies maintained by The Biomedical Services Unit of Leicester University) shown to have elevated arterial pressure in comparison to the age matched normotensive control colony. Clonal colonies of cells exhibiting smooth muscle morphology were combined to generate cultures 100% positive for smooth muscle actin immunofluorescence. The results described here were replicated in 2 separate cultures. Cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, penicillin (100 IU/ml), streptomycin (10 μg/ml), and glutamine (27 mg/ml), with 5% CO2, 95% air. Cells were used between passages 8 and 14, in 24-well plates for AII procedures other than the fast protein liquid chromatography separations, when the cells were from 175-cm² flasks.

**Cell Stimulation and Lysis**—Cells at 80–90% confluence were maintained serum-free for 24 h, washed 3 times with balanced salt solution (125 mM NaCl, 5.4 mM KCl, 16.2 mM NaHCO3, 30 mM HEPES, 1 mM NaH2PO4, 0.8 mM MgSO4, 1.8 mM CaCl2, 5.5 mM glucose; buffered to pH 7.4 with NaOH and gassed with 95% O2, 5% CO2) at 37°C, and then incubated with the indicated concentrations of agonist/inhibitor solution made up in balanced salt solution for the given time. Stimulations were terminated by the addition of liquid nitrogen. Cells were then lysed in homogenizing buffer (20 mM Tris, pH 7.4, 2 mM EDTA, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 1 μg/ml pepstatin A, 50 μM sodium fluoride, 2.5 mM sodium orthovanadate, 62.5 μM β-glycerophosphate, 1 μM phenylmethylsulfonyl fluoride, and 0.1% Triton X-100), scraped, sonicated for 10 min, and spun at 14,000 × g for 10 min at 4°C. Supernatants were then used immediately.

**Peptide Kinase Assay**—For measurement of MAPK activity in cell extracts, we have developed a peptide kinase assay based upon phosphorylation of the nonapeptide APRTPGGGR. This peptide has been designed specifically as a substrate for MAPK activity (23). A 10-μl aliquot of each supernatant or column fraction was incubated for 20 min at 30°C in the presence of 15 μl of assay buffer containing (final concentrations) 25 mM magnesium chloride, 1 mM of the substrate peptide, and 50 μl ATP/[γ-32P]ATP (1 μCi/tube). The reaction was terminated by the addition of 20% trichloroacetic acid. A 40-μl aliquot of each sample was then spotted onto P81 phosphocellulose paper, washed extensively in 75 mM phosphoric acid, and counted for [32P] incorporation.

**Resource Q Chromatography**—175-cm² flasks were extracted into 1.2 ml of homogenizing buffer. 1 ml of the resulting supernatant was then loaded onto a Pharmacia Resource Q anion-exchange column which had been previously equilibrated with 20 mM Tris, pH 7.4, 10 mM β-glycerophosphate, 1 mM EDTA, and 0.1 mM sodium orthovanadate. Elution was carried out with a linear gradient from 0 to 0.5 M NaCl in the same buffer at a flow rate of 1 ml/min. Fractions were collected and immediately assayed for peptide kinase activity (as described). The remainder of each fraction was then precipitated using trichloroacetic acid (0.5 M final concentration). After a 5-min spin of 14,000 × g for 4°C, the supernatants were discarded and the pellets were re-suspended in 50 μl of 2 × SDS sample buffer. These were then boiled for 5 min and probed for phospho-MAPK antiserum.

**Western Blots**—Cells were separated by SDS-polyacrylamide gel electrophoresis on a 10% gel. Western blotting was done with antiphospho-MAPK antiserum (1:500 dilution). Phospho-MAPK immunoreactive bands were visualized using the Amersham ECL procedure and quantified by densitometric analysis. Our results, from Western blots of bacterially expressed p44 MAPK in both phosphorylated and unphosphorylated forms, and Western blots of extracts of both endothelial cells and smooth muscle cells showed that the antiserum recognizes both p42 and p44 forms of tyrosine-phosphorylated MAPK. Non-tyrosine-phosphorylated proteins were not recognized, as determined by lack of immunoreactivity to the negative control unphosphorylated recombinant protein, and lack of immunoreactivity to cell extracts from unstimulated samples which showed clear immunoreactive p42 and p44 bands when using an antiserum against the unphosphorylated forms of MAPK.

**Incorporation of [3H]Thymidine**—Cells at 80–90% confluence were maintained serum-free for 24 h, followed by a 30-min preincubation with PD 098059 where appropriate, a 1-h stimulation with the agonist (and PD 098059 where appropriate), and then a 19-h incubation with no agonist in serum-free medium. [3H]Thymidine (0.074 MBq ml⁻¹) was then added followed by a further 4-h incubation. The cell monolayer was then placed on ice and washed sequentially with balanced salt solution, ice-cold 5% trichloroacetic acid, and ethanol, taken up into 0.1 M NaOH and scintillation counted. 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.

**Assay for Phospholipase D Activity**—PLD activity in intact cells was measured as described in Refs. 24 and 25. Briefly, cells just at confluence in 24 wells were labeled for 24 h at 37°C in a phosphate-free balanced salt solution with [32P]orthophosphoric acid (0.25 MBq ml⁻¹). Preincubations for 10 min included butanol added to the labeling medium at a final concentration of 50 mM, following which the medium was removed, and stimulation in balanced salt solution 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 [32P]-labeled phosphatidylbutanol and phosphatidic acid were separated from other phospholipids by thin layer chromatography on oxalate-coated silica plates developed in ethyl acetate, acetic acid, 2:2:1 (v/v/v). The location of phosphatidic acid and phosphatidylbutanol was established using 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 (24, 25), in which case the agonist-stimulated counts in the phosphatidylbutanol spot are an index of PLD activity.

**Phospholipase C Assay**—Cells at 80–90% confluence, in 24-well multwells, were labeled overnight with myo-[2-3H]inositol (1 μCi ml⁻¹, 0.5 ml/well) in medium M199 with 25 IU ml⁻¹ penicillin, 25 μg ml⁻¹ streptomycin, at 37°C in 5% CO2. To the 0.5 ml of labeling medium was added 50 μl of 11-fold final concentration of LiCl (110 mM) in balanced salt solution. After further incubation for 15 min with agonists, total [3H]inositol (poly)phosphates were extracted and separated on small Dowex-1 (Cl⁻) columns.

**RESULTS**

**Effect of MEK Inhibitor PD098059 on AII Stimulation of MAPK**—We investigated the effect of the recently described MEK inhibitor PD 098059 (20) on AII stimulation of MAPK tyrosine phosphorylation and activity in the cultures of SHR-derived RASM cells. The assay was the phosphorylation of a nonapeptide which is a preferential substrate for MAPK (23). Fig. IA shows the chromatography of an extract from stimulated cells on a Resource Q column, followed by assay for kinase activity. Two peaks were routinely observed (see also Fig. 2), consistent with the elution pattern seen by others when measuring stimulated MAPK activity. There were no detectable peaks of activity in the unstimulated cells. When the same fractions were subject to immunoblots with an antibody which recognized specifically the tyrosine-phosphorylated form of MAPK it was seen that the 2 peaks of kinase activity co-eluted with p42 and p44 bands of phosho-MAPK immunoreactivity (Fig. IB). The use of the phospho-MAPK immunoblot specifically measures the tyrosine phosphorylation which is required for the activation of MAPK, achieving in a single immunoblot procedure a result which has previously required sequential immunoprecipitation with p42 and p44 antibodies and testing the phosphoprotein Western blots. There was no detectable phosho-MAPK immunoreactivity in chromatography fractions of extracts of unstimulated cells. These results show that MAPK, PLD, and AII-stimulated Mitogenesis
stimulates both p42 and p44 forms of MAPK. Fig. 2 also shows that when the AII stimulation was in the presence of PD 098059 there was a partial (10 μM) or complete (100 μM) inhibition of activity in both stimulated peaks. Crude cell extracts (without fast protein liquid chromatography separation) also showed increased activity with stimulated cells (2.39 ± 0.16 fold/basal, n = 6), and in Fig. 3A it can be seen that this was inhibited in a concentration-dependent manner by PD 098059. Pooling across separate experiments showed (for the p42 MAPK band): unstimulated control, 0.019 ± 0.008; AII (100 nM), 0.404 ± 0.061; PD 098059 (100 μM), 0.031 ± 0.006; AII + PD 098059, 0.108 ± 0.008 (data are OD units/μg of protein, mean ± S.E., n = 4).

Effect of PD 098059 on [3H]Thymidine Incorporation—The results presented above established that the MEK inhibitor PD 098059 was able to inhibit the MAPK response to AII stimulation in the SHR-derived RASM cells. Fig. 4 shows that increasing concentrations of PD 098059 also inhibited the AII-stimulated increase in incorporation of [3H]thymidine. The IC50 was 17.8 ± 3.1 μM (n = 3), with complete inhibition of the response with 100 μM PD 098059.

Effect of PD 098059 on the PLC Response to AII Stimulation—To investigate the influence of the MEK inhibitor on other responses of the cell to AII stimulation we monitored the accumulation of total [3H]inositol (poly)phosphates as an index of PLC activity. Fig. 5 shows that the PLC response to AII stimulation was unaffected by concentrations of PD 098059 up to 100 μM. This indicates that PD 098059 was not interfering with the process which begins with agonist binding and proceeds through G protein coupling to the activation of PLC.

Comparison of Responses to AII and Phorbol Myristate Acetate (PMA)—In preliminary experiments we observed that the protein kinase C stimulating phorbol ester PMA was also capable of activating MAPK. To further characterize the relationship between MAPK and mitogenesis, we compared the responses when stimulation was with AII and PMA. Fig. 6 shows that maximally effective concentrations of AII and PMA (determined by concentration-response curves measuring the peptide kinase activity in the crude extracts, data not shown) gave an essentially identical size and time course of response using the peptide kinase assay. Despite this, the data in Fig. 7 shows
that, while AII generated a substantial increase in \[^3H\]thymidine incorporation (Fig. 7A), PMA gave a small (Fig. 7B) response which was nonsignificant when pooled across experiments.

This outcome showed that, while PMA stimulates MAPK, it cannot elicit an increase in DNA synthesis. To ask whether PMA activates a pathway that inhibits the mitogenic response to AII, we first stimulated the cells for 1 h with AII as described above, and then investigated the effect of adding PMA for the following 19 h prior to addition of \[^3H\]thymidine. Over three experiments (each in quadruplicate), the stimulation caused by 100 nM AII followed by 100 nM PMA for 19 h was 1.14 ± 0.19-fold the stimulation in the absence of PMA. This shows that the presence of PMA following addition of AII has no significant effect on the stimulation of DNA synthesis caused by AII.

Taken together the results with PMA indicated that the size and duration of the MAPK response was not, taken alone, sufficient to predict the size of the stimulation in thymidine incorporation, suggesting that another intracellular signaling event was required for a full response.

A Role for PLD—We have previously provided evidence that implicates PLD in the signaling pathway from the AII receptor to mitogenesis in the SHR-derived cells (11). The activation of PLD generates, as its primary lipid product, phosphatidic acid; it may be this, or a metabolite of phosphatidic acid, which is the mitogenic product of PLD. However, in the presence of butanol the primary product is not phosphatidic acid, but phosphati-
dybutanol. As well as providing the basis for an assay for PLD (24, 25), this provides a method for the diversion of the PLD product and thus the inhibition of events dependent on the PLD activity. Here, we exploit the dependence of this transphosphatidylation reaction on the presence of a primary alcohol: while 1-butanol will participate, tertiary butanol will not. This is shown with these cells and AII stimulation in Fig. 8, in which it can be seen that formation of AII stimulated phosphatidylbutanol occurred in the presence of 1-butanol but not tertiary butanol. At concentrations of 30–50 mM, 1-butanol causes essentially all the lipid product of the PLD reaction to be diverted to phosphatidylbutanol (24, 25), resulting in the loss of 40–50% of the \[^3H\]thymidine response of SHR-derived cells to AII (11); by contrast, the PLC response was unaffected by 1-butanol. The use of tertiary butanol enables us to provide a further control for this experiment, since it would be anticipated that both isoforms of butanol would have the same nonspecific effects, but to differ in the interference with PLD mediated responses. We compared the effect of the two forms of butanol on AII-stimulated SHR-RASM \[^3H\]thymidine incorporation. The response to 100 nM AII in the presence of 50 mM butanol, expressed as percentage of that in the absence of butanol, was 61.5 ± 7.1 for 1-butanol and 92.6 ± 4.8 for tertiary butanol. Therefore, at concentrations of 1-butanol which caused a loss of about 40% of the \[^3H\]thymidine response (as in our earlier report), the presence of tertiary butanol was without significant effect.

To investigate the relationship between AII-stimulated MAPK, PLD, and \[^3H\]thymidine responses, we studied the PLD response to stimulation with AII and PMA in the absence and presence of PD 098059. Fig. 9 shows, as reported earlier (11), that AII elicited a substantial PLD response, and addi-
tionally shows that this response was not attenuated by concentrations of PD 098059 sufficient to abolish the \[^{3}H\]thymidine response. This tells us that the PLD response is not downstream of MEK/MAPK, and that the inhibition of stimulated \[^{3}H\]thymidine incorporation by PD 098059 is not due to attenuation of the PLD response. Fig. 9 also shows that PMA produced little or no PLD response, a situation unaffected by the presence of PD 098059. Pooled over separate experiments, expressed as counts/min/μg of protein of \[^{32}P\]phosphatidylbutanol (mean ± S.E., n = 3) from an experiment representative of three.

\[\text{DISCUSSION}\]

The signal transduction pathways which lead from stimulation of a 7-transmembrane heterotrimeric G protein-coupled receptor to a mitogenic response are not clear. Several previous publications have shown that this superfamily of receptors can, in many instances, activate tyrosine protein kinases and the MAPK cascade. These are the events that have been implicated in the mitogenic response to those classical growth factors which act on single transmembrane tyrosine kinase receptors. However, it has proved difficult to directly investigate the significance of the MAPK pathway to the mitogenic response elicited by activation of G protein-coupled receptors. These receptors classically produce changes in cyclic AMP, PLC, and PLD, cytosolic Ca\(^{2+}\), the protein kinase C family and diverse protein kinase cascades, all of which are putative contributors to the pathways to mitogenesis. The importance, if any, of MAPK in the mitogenic response has not been clear.

In contrast to this, there has been considerable elucidation of the mechanisms by which G protein-coupled receptors may activate tyrosine kinases and the MAPK cascade. Earlier indications showed that there were pathways both dependent on and independent of the stimulation of protein kinase C and elevation of cytosolic Ca\(^{2+}\) (26, 27), implying that, while a PLC linked receptor can activate the tyrosine kinase/MAPK cascades through PKC and Ca\(^{2+}\), there is also a route from the receptor which is independent of PLC. Further reports (e.g. Ref. 28) showed that Ras- and Raf-dependent and independent pathways exist. Significantly, it has become apparent that there is widespread evidence for direct G protein \(\beta\gamma\)-subunit activation of the Ras/MAPK cascade. However, it has also been proposed that PLC linked activation of MAPK may be by sequential activation of a specific tyrosine kinase and thus of Ras and Raf; this tyrosine kinase-dependent activation of Ras and Raf has also been proposed for \(\beta\gamma\)-subunit activation of MAPK (32, 33). It is of importance for the present study to note that these diverse pathways proposed for the activation of MAPK by G protein-

**FIG. 8.** Accumulation of \[^{32}P\]phosphatidylbutanol (\[^{32}P\]P-But) in the presence of 1-butanol and tertiary butanol. Effect of stimulation with AII in the presence of 50 mM tertiary butanol (open bars) or 1-butanol (hatched bars). Data are mean ± S.E. pooled across three separate experiments each in triplicate.

**FIG. 9.** Stimulation of PLD by AII and PMA: effect of PD 098059. Cells were incubated for 20 min in the presence of 1-butanol with no stimulation (○) or with 100 nM AII (●) or PMA (■) and the concentrations of PD 098059 indicated. Data is mean ± S.E. pooled across three separate experiments each in triplicate.
coupled receptors all converge downstream of Raf onto the MAPK activating kinase, MEK. Further studies provide evidence that cyclic AMP also plays a significant role in the regulation of MAPK by G protein-coupled receptors (34, 35).

These pathways are involved in the response to AII of smooth muscle and other cell types. AII was shown to enhance tyrosine phosphorylation in glomerular mesangial cells by protein kinase C-dependent and -independent routes (36). A role for Ca²⁺ has been documented (37), and the activation of MAPK demonstrated (16, 38). The link with Ras, and evidence that pp60⁵⁺⁺ is required for the AII stimulation of MAPK in vascular smooth muscle cells (18), provided further detail. This work shows that AII activation of MAPK proceeds via tyrosine kinase and activation of the Ras-Raf pathway as shown in other systems, and that downstream of Raf is the MEK phosphorylation and activation of MAPK. This involves the unusual dual kinase activity of MEK, with sequential tyrosine and threonine phosphorylations (39), resulting in the stimulation of the kinase activity. Despite the progress made by these studies, the role of AII-stimulated MAPK in vascular smooth muscle cells has not been demonstrated.

In the present study we initially set out to ask two questions: first, is MAPK required for the mitogenic response to AII of vascular smooth muscle cells; second, is the activation of MAPK, with appropriate magnitude and duration, sufficient for a mitogenic response, or are other pathways necessary? Using an inhibitor of MEK/MAPK activation (20, 40), we have shown, using a mitogenically responsive vascular smooth muscle cell line, that the AII-stimulated tyrosine phosphorylation and activation of both p42 and p44 forms of MAPK can be inhibited by PD 098059 in a concentration-dependent manner. We have then shown that the enhanced thymidylate incorporation in response to AII can be inhibited by PD 098059 with a similar concentration dependence as for the inhibition of the activation of MAPK. The IC₅₀ for these inhibitors is similar to those previously described for the inhibition of MEK/MAPK activation by PD 098059 (40). This study confirms that, in the intact vascular smooth muscle cell, AII stimulation of MAPK proceeds, as expected, through a PD 098059-sensitive MEK.

More important, the study leads to the conclusion that MAPK is an absolute requirement for a mitogenic response to AII in the SHR-RASM cells. Several considerations support this conclusion. First, we have shown that the [³H]thymidine response to AII is accompanied by a subsequent increase in cell number, showing that there is a true mitogenic response. Secondly, the design of the [³H]thymidine procedure enables a relatively short exposure to both agonist and inhibitor, unlike a conventional design for a thymidine incorporation study in which the inhibitor would have to be in contact with the cells for many hours. Third, we have shown that other responses to AII (PLC and PLD) are not affected by the MEK inhibitor. This shows quite clearly that the inhibitor does not interfere with the agonist recognition or signal transduction pathways through the G protein to the phospholipases. The last two points mitigate the suggestion that the effects of PD 098059 on the mitogenic response are due to actions other than inhibition of MEK activation, consistent with earlier reports establishing the high degree of specificity of this inhibitor.

While these results support the view that MAPK is required for the mitogenic response to AII, the results do not support the view that this is sufficient to generate a mitogenic response. This is clear from the stimulation of the cells with PMA, which gave an activation of MAPK which had the same magnitude and time course as that seen with AII. However, PMA did not give a stimulation of [³H]thymidine incorporation. This provides a powerful argument against the view, recently put forward, that it is the duration of the MAPK activation which determines whether it will result in a mitogenic response (7, 19).

An alternative interpretation of these results was that the presence of PMA exerted an inhibitory influence on the mitogenic consequences of MAPK activation. This hypothesis might explain the lack of mitogenic response to PMA despite activation of MAPK, and could be tested by the simultaneous presence of PMA with AII. However, addition of PMA with AII has multiple effects on early signaling events, including attenuation of the PLC response, making the interpretation of such a study rather difficult. An alternative strategy was adopted, of adding the PMA at the end of the 1-h stimulation with AII: if PMA generated a change which inhibited a mitogenic response to activated MAPK, this should have attenuated the mitogenic response to AII when PMA was added in the period between exposure to agonist and measurement of thymidine incorporation. No such attenuation was seen, indicating that the lack of mitogenic response to PMA is unlikely to be due to the recruitment of an inhibitory pathway.

These observations, taken together, suggest that inputs other than MAPK activation are required to elicit a mitogenic response to AII. Elevated cytosolic Ca²⁺ is a clear candidate for such a signaling input. Here, we have directly considered a role for another such candidate, PLD.

There has been considerable interest in PLD as a component in mitogenic signal transduction, with some specific evidence for such a role in vascular smooth muscle cells (see Ref. 21, for review). The lack of a specific inhibitor for PLD has hindered direct examination of this hypothesis; however, we have recently shown that if we divert the product of the PLD reaction away from phosphatidic acid by the presence of 30–50 mM 1-butanol, then we lose close to half of the thymidine incorporation response of SHR-RASM cells to AII (11). Here we provide evidence that this is a specific effect with respect to the interference with the transphosphatidylation reaction of PLD, since we show that the same concentrations of tertiary butanol will neither participate in the PLD reaction nor inhibit AII stimulated thymidine incorporation. This provides strong support for the notion that PLD is required for a full mitogenic response. We also show here that PMA is not able to stimulate a PLD response in these SHR-RASM cells. This is significant, since PMA also does not stimulate thymidine incorporation, so this observation is consistent with the notion that both MAPK and PLD are required for the full mitogenic response. Further supportive evidence comes from studies with stimulation of vascular smooth muscle cell mitogenesis with platelet-derived growth factor, where both MAPK and PLD were activated and a causative role for PLD was proposed (41).

To summarize, AII is able to activate MAPK and PLD, and generates a mitogenic response dependent on both. Contrasting with this, PMA gives an identical stimulation of MAPK but no activation of PLD, and is not able to elicit a mitogenic response. In conclusion, these results provide strong evidence for an absolute requirement for MAPK in the mitogenic response to AII of cultured vascular smooth muscle cells, but show that in addition other mitogenic signaling pathways must be activated, one of which is PLD.

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