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Angiotensin II responses of vascular smooth muscle cells from hypertensive rats: enhancement at the level of p42 and p44 mitogen activated protein kinase

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1 Stimulation of the AT_1 receptor by angiotensin II (AII) gives a larger mitogenic response in vascular smooth muscle cells from spontaneously hypertensive rats (SHR) compared to those from normotensive (WKY) controls. Here we investigated whether the p42 and p44 mitogen activated protein kinase (MAPK) pathway is differentially regulated in these cells by AT_1 receptors.

2 We showed that there is a similar level of p42 and p44 MAPK immunoreactivity in the SHR and WKY derived cells.

3 However, by use of an antiserum specific for the tyrosine phosphorylated form of MAPK, and an assay with a nonapeptide MAPK substrate, we showed that AII (100 nM)-stimulated phosphorylation and activation of $p42^{mapk}$ and $p44^{mapk}$ are enhanced in the SHR derived cells.

4 This increased MAPK activity in SHR derived cells was also seen on protein kinase C activation with 100 nM phorbol myristate acetate (PMA). The size and time course of the response to PMA was the same as that to AII in each cell type.

5 The protein kinase C inhibitor Ro 31-8220 attenuated the early (2 min) phase of AII stimulation of MAPK activity and the entire stimulation caused by PMA. At longer times of AII stimulation both $p42^{mapk}$ and $p44^{mapk}$ were activated by an Ro 31-8220-insensitive mechanism.

6 Agonist or PMA stimulation of MAPK activity was inhibited by the tyrosine kinase inhibitor genistein. AII stimulated tyrosine protein phosphorylation to a greater degree in SHR than WKY cells.

7 These results show that the MAPK response of SHR derived cells is increased over that of WKY cells by mechanisms independent of the enhanced stimulation of phospholipase C; amplification at the level of sequential protein kinase C and tyrosine kinase steps leads to the enhanced responsiveness of MAPK in the SHR derived cells.

Keywords: Angiotensin II; vascular smooth muscle; hypertension; protein kinase C; MAPK; spontaneously hypertensive rats (SHR)

Introduction

The renin-angiotensin system exerts its influence over vascular smooth muscle (VSM) cells by the action of angiotensin II (AII) on seven transmembrane AT_1 receptors (Peach, 1981; Sasaki *et al*., 1991; Murphy *et al*., 1991). These are coupled through a member of the G_q family of G proteins (Smrcka *et al*., 1991; Taylor *et al*., 1991) resulting in the activation of phospholipase C (PLC) and consequent elevation of inositol (1,4,5)trisphosphate, cytosolic Ca^{2+} and diacylglycerol (Griendling *et al*., 1986; Peach & Dostal, 1990). Furthermore, it has been shown that there are differences in these signalling cascades between VSM derived from spontaneously hypertensive rats (SHR) and those from normotensive control rats (WKY); the SHR derived cells show enhanced PLC and Ca^{2+} responses on stimulation with AII (Resink *et al*., 1989; Paquet *et al*., 1990; Osani & Dunn, 1992; Bunkenberg *et al*., 1992; Morton *et al*., 1995; Baines *et al*., 1996). Following from these signalling studies, AII receptors have also been shown to play a role in the regulation of cell division: in cell culture it is apparent that AII exerts only a weak or undetectable mitogenic effect on VSM derived from aortae of normotensive rats, but elicits a more substantial mitogenic response with SHR derived cells (Paquet *et al*., 1990; Bunkenberg *et al*., 1992; Butcher *et al*., 1993; Morton *et al*., 1995). We have exploited this differential mitogenic response in attempts to understand the mechanisms linking the AT_1 receptors to the control of VSM proliferation, providing evidence that an enhanced phospholipase D response may contribute to the enhanced mitogenic response of the SHR derived cells on exposure to AII (Morton *et al*., 1995; Wilkie *et al*., 1996).

However, the results presented in our earlier study also indicate that other, phospholipase D-independent, pathways are important in generating the differential mitogenic response of the SHR derived cells. Other studies have implicated tyrosine kinase pathways in the response of VSM to AII (e.g. Berk & Corson, 1997). We have shown that p42 and p44 forms of mitogen activated protein kinases (MAPK: also known as extracellular signal related kinases, or ERKs) are necessary but not sufficient for the mitogenic response of SHR derived cells (Wilkie *et al*., 1996). Here we provide a direct comparison of this AII stimulated MAPK cascade in the SHR and WKY derived cells, showing that the activation of MAPK is greater in SHR derived cells and providing some information relating to the mechanism of this enhanced response.

Methods

Cell preparation and culture

Cells were derived from SHR and normotensive control WKY colonies maintained by the Biomedical Services Unit of Leicester University. Aortae were taken from 12 week old animals after determination of blood pressure by the use of a tail cuff, and cells were prepared by the method described by Davies *et*

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al. (1991) and summarized in Morton *et al*. (1995). Cells were cultured in Dulbecco's modified Eagle's medium with 10% foetal calf serum, 100 i.u. ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin and 27 mg ml^{-1} glutamine, maintained at 37°C in 5% CO_2, 95% air. Cells were 100% positive for smooth muscle actin immunofluorescence. Quiescent cells (maintained serum free for 24 h) were used at passages 8–14.

Cell stimulation and lysis

Cells at 80% confluence were maintained serum-free for 24 h, washed 3 times with balanced salt solution (mM: NaCl 125, KCl 5.4, $NaHCO_3$ 16.2, HEPES 30, NaH_2PO_4 1, $MgSO_4$ 0.8, $CaCl_2$ 1.8 and glucose 5.5, buffered to pH 7.4 with NaOH and gassed with 95% O_2, 5% CO_2) at 37°C and then incubated with the indicated concentrations of agonist/inhibitor solution made up in balanced salt solution for the stated times. Stimulations were terminated by snap freezing the monolayer with liquid nitrogen. Cells were then lysed in homogenizing buffer (20 mM Tris pH 7.4, 2 mM EDTA, 10 $\mu g\ ml^{-1}$ leupeptin, 20 μM E-64 (*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane), 2 $\mu g\ ml^{-1}$ aprotinin, 1 μM pepstatin A, 50 mM sodium fluoride, 2.5 mM sodium orthovanadate, 62.5 mM β-glycerophosphate, 1 μM phenylmethylesulphonyl fluoride and 0.1% Triton X-100), scraped, bath sonicated for 10 min and spun at 14 000 *g* for 10 min at 4°C. Supernatants were then used immediately.

Western blots

Cells extracted from 80 cm^2 flasks were equalized for protein content by the Lowry protein assay (typically about 10 μg protein was used for each analysis), boiled for 5 min with Laemmli sample buffer and resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS/PAGE) on a 10% gel. After electrophoretic transfer to nitrocellulose membranes, the blots were blocked overnight in 10% dried milk (Marvel) in Tris buffered saline (20 mM Tris pH 7.6, 137 mM NaCl and 0.05% Tween-20). Western blotting was performed by incubating the nitrocellulose membranes in anti-MAPK antiserum (routinely a 1:20000 dilution of our anti-MAPK antiserum or a 1:5000 dilution of the anti-MAPK antiserum provided by P.J. Parker, each made up in 5% Marvel in Tris buffered saline) for 11/2 h. After washing off excess primary antibody, the membranes were incubated for a further hour with a secondary antibody conjugated to horseradish peroxidase. MAPK immunoreactive bands were visualized with the Amersham ECL procedure and quantified by densitometric analysis. An alternative was the use of an antiserum raised against the tyrosine phosphorylated form of $p44^{mapk}$, used at 1:1000 dilution with the same immunoblot protocol, to provide an index of the stimulation of MAPK phosphorylation (phospho-MAPK immunoreactivity). This phospho-specific MAPK antibody (New England Bioproducts Ltd.) was raised against a synthetic phospho-tyrosine peptide coupled to keyhole limpet haemocyanin, corresponding to residues 196 to 209 of human $p44^{mapk}$ (DHTGFLTEY(p)-VATRWC), which reacts with $p44^{mapk}$ and $p42^{mapk}$ only when phosphorylated at Tyr204, being unreactive with even 1 μg non-tyrosine phosphorylated MAPK. Detection was with ECL as described above. This protocol has been validated in recent publications (Wilkie *et al*., 1996; Patel *et al*., 1996). In other cases the monoclonal anti-phosphotyrosine antibody PY20 was used at 1:1000 dilution to probe for tyrosine phosphoproteins.

Nonapeptide kinase assay

For measurement of MAPK activity in cell extracts we have developed an assay (referred to here as the peptide kinase assay) based upon phosphorylation of the nonapeptide APRTPGGRR (Wilkie *et al*., 1996). This peptide has been designed specifically as a substrate for MAPK activity (Clark-Lewis *et al*., 1991). 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 μM ATP/[γ-^{32}P]-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 ^{32}P incorporation.

Resource Q chromatography

Flasks 175 cm^{-2} 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 0–0.5 mM NaCl in the same buffer at a flow rate of 1 ml min^{-1}. Fractions were collected and immediately assayed for peptide kinase activity (as described). The remainder of each fraction was then precipitated with 0.5 mM trichloroacetic acid. After a 5 min spin of 14000 *g* at 4°C, the supernatants were discarded and the pellets were re-suspended in 50 μl of 2 × Laemmli sample buffer. These were then boiled for 5 min and subject to Western blot analysis with antibodies for MAPK, phosphotyrosine and/or phospho-MAPK immunoreactivity.

Materials

Cell culture medium was purchased from GIBCO (Paisley, Scotland), except for foetal calf serum, which was from Advanced Protein Products Ltd (Birmingham, West Midlands, U.K.). The protein kinase C inhibitor Ro 31-8220 (bisindolylmaleimide IX) was a generous gift of Roche Products Ltd, (Welwyn, U.K.). The nonapeptide substrate APRTPGGRR for the MAPK assay was synthesized by the peptide synthesis laboratory at Leicester University. [γ-^{32}P]-ATP and the horseradish peroxidase conjugated secondary antibodies were purchased from Amersham, (Bucks, U.K.). Primary antibodies against MAPK were either raised in our own laboratory against a hexyl-histidine fusion protein derived from the entire cDNA sequence of ERK2 (extracellular signal regulated kinase 2, from M. Cobb, Department of Pharmacology, University of Texas, U.S.A.) or were a kind gift from P.J. Parker, (ICRF, London). Antibodies to phosphotyrosine (PY20) and phosphotyrosine phosphorylated MAPK were from ICN (Oxfordshire, U.K.) and New England Bioproducts Ltd, (Herts, U.K.), respectively.

Statistics

Results are expressed as means ± s.e.mean, and comparisons were by Student's *t* test, performed on an Oxstat statistics package (Microsoft Corporation, Reading, U.K.). Two tailed *P* values under 0.05 were considered significant.

Results

Immunoreactivity in SHR vs WKY cell extracts

Western blots with anti-MAPK antiserum indicated that there was a similar level of immunoreactivity in SHR and WKY cell extracts (e.g. Figure 1a). Over a series of 7 experiments, in each of which the SHR and WKY cells were analysed in parallel, and the conditions of ECL development standardized, the immunoblots were scanned for each of the 2 molecular weight bands. Data are expressed as means ± s.e.mean of optical density units mg^{-1} protein. The results were: SHR $p42^{mapk}$, 94.8 ± 11.6; WKY $p42^{mapk}$, 94.5 ± 13.6; SHR $p44^{mapk}$, 6.5 ± 0.4; WKY $p44^{mapk}$, 6.7 ± 0.7 (P = NS).

Further immunoblot analysis used antisera specific for tyrosine phosphorylated MAPK (Figure 1b) and tyrosine phosphorylated phosphoproteins (Figure 1c). Stimulated activity (100 nM AII for 2–5 min) was higher in SHR than WKY extracts (Figure 1b). Pooled across 5 experiments the stimulated phospho-MAPK immunoreactivity of WKY cells, expressed as a % of that of SHR cells, was $46.1 \pm 9.4\%$ ($p42^{mapk}$) and $53.7 \pm 9.5\%$ ($p44^{mapk}$) ($P < 0.001$ for both, compared to SHR cells). By using antiserum for tyrosine phosphoproteins, immunoreactivity could be detected in all extracts. However, it was substantially increased by stimulation (100 nM AII for 2–5 min), and again was higher in SHR than WKY cells. The stimulated phosphoproteins were particularly apparent in a large number of proteins above a molecular weight of approximately 90 kD, as shown in Figure 1c. Overexposure of these blots resulted in the appearance of phosphotyrosine bands corresponding to the p42 and p44 MAPK proteins in the stimulated samples only (Figure 1c). Again there was greater phosphorylation in stimulated extracts from SHR than WKY derived cells.

Analysis by peptide kinase assay

We have utilized an assay procedure based upon phosphorylation with [γ-^{32}P]-ATP of a nonapeptide substrate derived from the sequence of a myelin basic protein (MBP) fragment and which is optimized as a substrate for $p44^{mapk}$ kinase activity and shows some selectivity as a substrate for MAPK activity over other protein kinases (Clark-Lewis *et al*., 1991; Wilkie *et al*., 1996). To determine the relationship between this and MAPK activity we analysed the cell extracts by Resource Q chromatography, followed by kinase assay (by the procedure described above) and Western blots (with anti-MAPK antisera). Assay of chromatography fractions from SHR derived cells revealed the presence of two peaks of enzyme activity, in both stimulated and unstimulated samples (Figure 2). Both peaks were considerably elevated by stimulation with 100 nM AII for 5 min. The two peaks of enzyme activity corresponded with the elution of MAPK immunoreactivity. While the results in Figure 2 show that the first peak contained both p42 and p44 MAPK immunoreactivity, when the immunoblots were developed with antiserum to the tyrosine phosphorylated form of MAPK the first peak contained only phospho-p42 activity, and the second only phospho-p44 activity (not shown). This indicates that the two peaks of enzyme activity do represent a separation of p42 and p44 activated MAPK. This pattern of 2 peaks is similar to that obtained in other studies on MAPK activity, including experiments on AII-stimulated VSM cells (Ishida *et al*., 1992) in which MBP was the

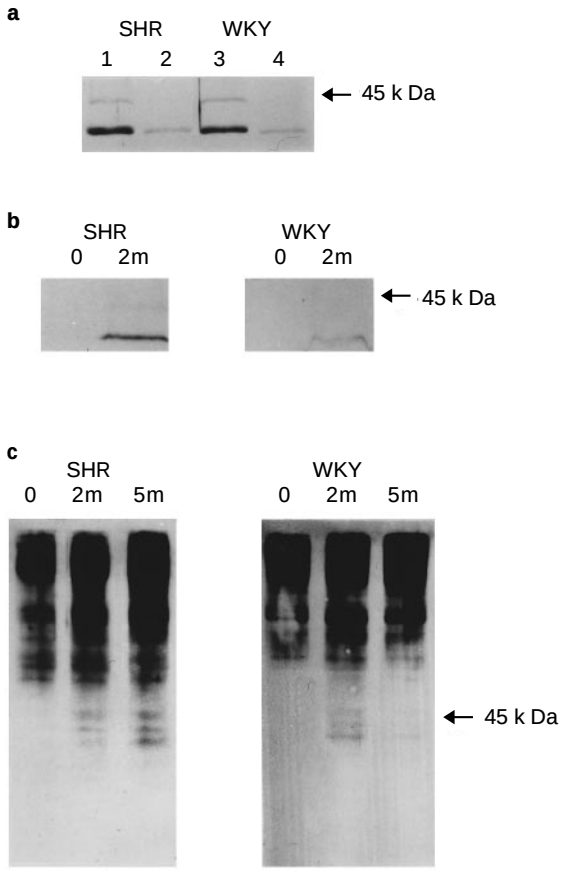

Figure 1 Comparison of MAPK, phospho-MAPK, and phosphotyrosine immunoreactivity in SHR and WKY derived cells by western analysis of cell homogenates. (a) Immunoblot obtained with our anti-MAPK antiserum, on SHY and WKY derived cell homogenates at two different dilutions of the primary antiserum (dilutions were 1:20,000 for lanes 1 and 3, and 1:40,000 for lanes 2 and 4). (b) Phospho-MAPK immunoblots of SHR and WKY derived cell homogenates, after stimulation with 100 nM AII for times indicated. (c) Anti-phosphotyrosine immunoblots of SHR and WKY cell extracts stimulated with 100 nM AII for times indicated. In each case the proteins in the WKY and SHR extracts were equalized before separation on the SDS-PAGE gel. Results are representative of at least 3 separate experiments in each case.

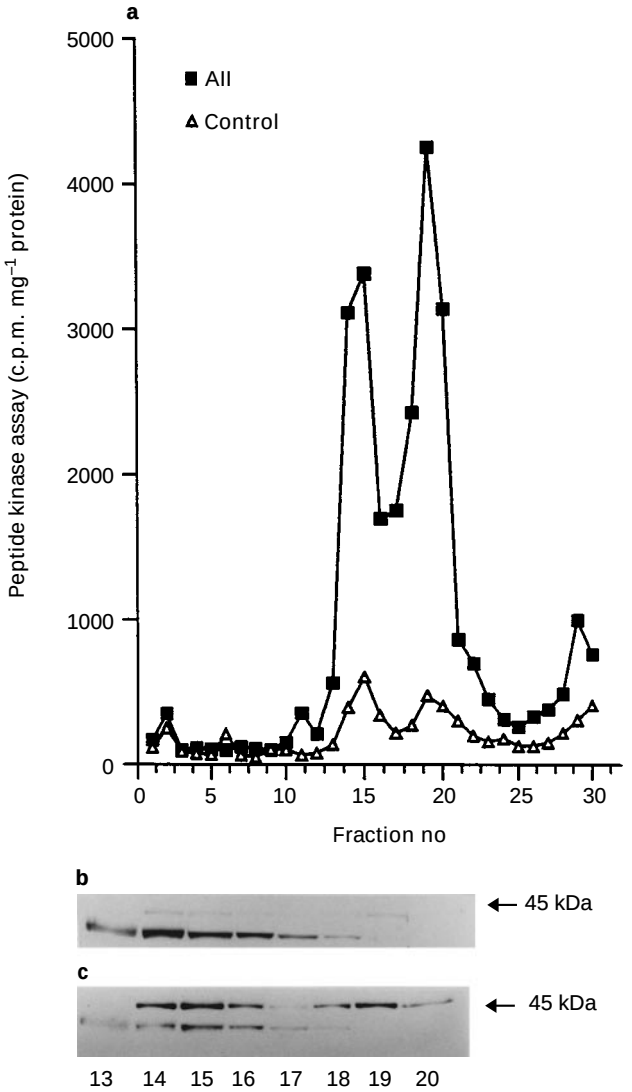

Figure 2 Analysis of extracts of SHR derived cells by Resource Q chromatography. (a) Extracts of AII (100 nM for 5 min)-stimulated and unstimulated (control) cells were separated and aliquots analysed for peptide kinase activity; fractions from the stimulated sample were analysed by immunoblots with our anti-MAPK antiserum (b) and that supplied by P.J. Parker (c). Representative of 3 experiments.

substrate. Taken together these results indicate that the agonist-stimulated activity seen with the peptide kinase assay principally measures both the p42 and p44 isoforms of MAPK.

Involvement of protein kinase C and comparison of SHR and WKY derived cells

Figure 3 illustrates the response to increasing concentrations of AII (a) and PMA (b) with a 5 min stimulation period, followed by extraction and assay. Pooled across experiments the EC_{50} for AII was 4.76 ± 0.32 nM and for PMA was 4.98 ± 0.52 nM ($n=4$). In WKY cells, the EC_{50} for AII was 5.98 ± 0.71 nM and that for PMA was 5.88 ± 0.56 nM ($n=4$). The time course of stimulation of the peptide kinase activity showed a transient increase, with a peak at 4–5 min for the SHR cells and at about 3 min for the WKY cells. This was true for both AII and PMA stimulation, as illustrated in Figure 4. The rates of decline in peptide kinase activity were similar in both cell types.

Figure 4 also shows that the response of the WKY cells was smaller than that of the SHR cells for stimulation with PMA or AII. The time course of these responses was determined 4 times, and data taken from the peak of the response, pooled across experiments, is presented in Table 1. The results of this analysis showed that the SHR cells had a higher peptide kinase activity than WKY cells whether unstimulated, or stimulated with AII or PMA. Figure 4 and Table 1 demonstrate that in both WKY cells and SHR cells the stimulation of peptide kinase activity by AII was the same size as the stimulation by PMA. On Resource Q separation of AII stimulated cells the WKY derived activity separates into two peaks corresponding to those for the SHR extracts; both peaks were similarly lower in the WKY cells (not shown). Pooled across 4 experiments and expressed as % of the value for the SHR cells, the activity of the WKY cells was, for the first peak, $53.3 \pm 2.2\%$, and for the second peak, $49.8 \pm 3.2\%$ ($P<0.001$ compared to SHR values for both). Combined with the analysis of phospho-

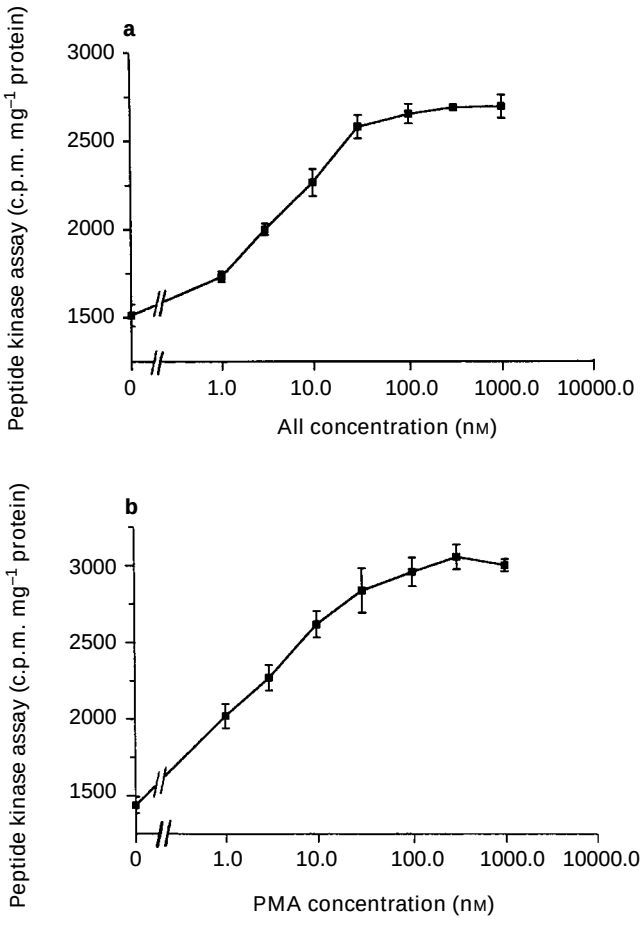

Figure 3 Stimulation of SHR derived cells by increasing concentrations of AII or PMA, and analysis of cell extracts by peptide kinase assay. AII (a) or PMA (b) were present for 5 min at the concentrations shown. The experiment was undertaken in triplicate with data expressed as mean from a single experiment representative of 4; vertical lines show s.e.mean.

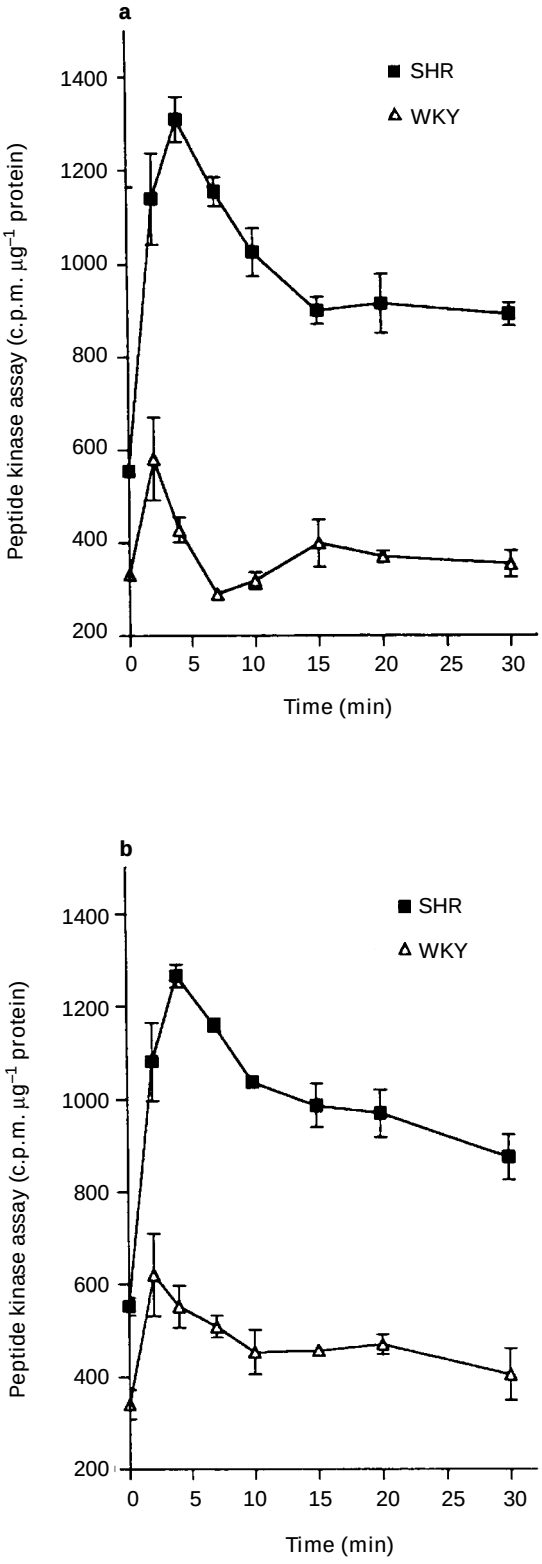

Figure 4 Time course of stimulation of SHR and WKY derived cells with 100 nM AII (a) or PMA (b): analysis by peptide kinase assay. Data are from a single experiment carried out in triplicate, expressed as mean with vertical lines indicating s.e.mean, and are representative of 4 separate experiments. Data pooled across experiments are presented in Table 1.

MAPK activity seen in Figure 1b these results demonstrate that the AII-stimulated MAPK activity was higher in SHR cells than in WKY cells for both the p42 and the p44 forms.

Figure 5 shows the effect of the relatively selective protein kinase C inhibitor Ro 31 8220 (compound 3 in Davis *et al.*, 1989) on stimulation of the kinase activity in extracts of cells stimulated with either AII or PMA. The presence of Ro 31-8220 caused a profound inhibition of the response to PMA at all times of stimulation. In contrast to this, the effect of Ro 31-8220 on AII-stimulated activity resulted in inhibition at earlier, but not later, times of stimulation (Figure 5). This was true for each individual experiment undertaken. Data pooled across experiments on SHR cells (c.p.m. μg^{-1} protein, $n=7$) showed that the response to stimulation with 100 nM AII for 2 min was 3038 ± 482 in the absence of Ro 31 8220, but was 921 ± 316 in the presence of 10 μM Ro 31-8220 ($P<0.01$). However, when the responses at 10 min were compared, these were 2651 ± 516 in the absence and 2555 ± 452 in the presence of Ro 31-8220 (not significantly different). In parallel experiments we showed that with 2 min stimulation of SHR cells with 100 nM AII, followed by Resource Q chromatography, Ro 31-8220 attenuated both peaks of kinase activity and attenuated the tyrosine phosphorylation of both p42 and p44 MAPK bands (not shown).

Effects of the tyrosine kinase inhibitor genistein

In an attempt to understand further the relationship between the pathways for stimulation by PMA and AII, we used inhibition of tyrosine kinases by genistein. Figure 6 shows that genistein is effective in inhibition of both AII and PMA stimulated peptide kinase activities at all time points in extracts of SHR derived cells. The peak response was reduced to a mean of $29.5 \pm 1.1\%$ for PMA stimulation and $25.4 \pm 1.1\%$ for AII stimulation in the presence of genistein ($n=5$). The pattern of residual activity was identical for either stimulus (Figure 6) in each experiment undertaken. In other experiments we have observed that the AII stimulation of phospho-MAPK activity in SHR derived cells was also severely attenuated by genistein (data not shown), confirming the effect of this tyrosine kinase inhibitor on AII stimulation of MAPK.

Discussion

In previous studies it has been shown that phospholipase responses to stimuli are larger in SHR cells compared to WKY controls. For example, there is an enhanced PLC response to AII in SHR cells, leading to an augmented generation of inositol (poly)phosphates and rise in cytosolic Ca^{2+} (Nabika *et al.*, 1985; Resink *et al.*, 1989; Paquet *et al.*, 1990; Bukoski, 1990; Osani & Dunn, 1992; Bendhack *et al.*, 1992; Morton *et al.*, 1995; Baines *et al.*, 1996). There is also an enhanced AII-stimulated phospholipase D response, and evidence has been presented (Morton *et al.*, 1995; Wilkie *et al.*, 1996) that this plays some role in the elevated mitogenic response to AII seen in the SHR cells (Paquet *et al.*, 1990; Bunkenberg *et al.*, 1992). It has been shown that there are no differences in number and

Table 1 Comparison of activity in peptide kinase assay in AII and PMA stimulated extracts of SHR and WKY derived cells

	WKY	*SHR*
Basal ($n=8$)	360 ± 19.9	622 ± 35.9*
AII (100 nM) ($n=4$)	436 ± 79	1177 ± 236**
PMA (100 nM) ($n=4$)	476 ± 78	1082 ± 234**

Data are mean $\pm$ s.e.mean, c.p.m. μg^{-1} protein of ^{32}P phosphorylated peptide, taken from the peak response obtained on time course experiments, pooled across experiments (each in triplicate). Stimulated values shown are the change in peptide kinase activities from the basal value. Significance of difference between SHR and WKY values. *$P<0.001$; **$P<0.05$ (Student's t test).

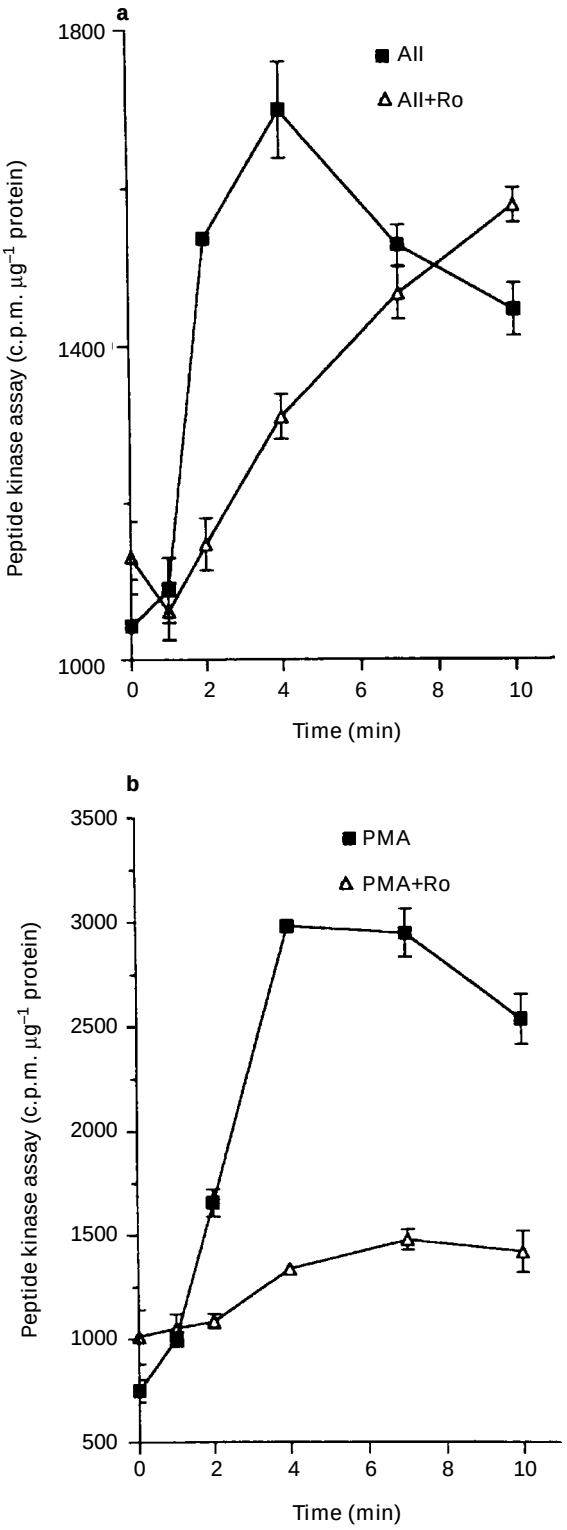

Figure 5 Time course of AII and PMA stimulated peptide kinase activity in SHR derived cells in the presence and absence of Ro 31-8220. AII and PMA were present at 100 nM, with Ro 31-8220 present at 10 μM for a 10 min preincubations as well as the 10 min stimulation period. Data are mean, with vertical lines indicating s.e.mean, from a single experiment carried out in triplicate, representative of 7, with pooled data presented in the text.

affinity of AII receptors between the cell types (Bolger *et al*., 1990; Osani & Dunn, 1992).

To study p42 and p44 MAPK activity we used a nonapeptide kinase assay. This assay has been characterized in earlier studies, which showed that the majority of all AII-stimulated activity comprises p42 and p44 forms of MAPK. Using this assay we showed that the activity was greater in SHR derived cells than in WKY cells. This was true with extracts from both AII and PMA stimulated cells. Independent confirmation that stimulation of MAPK was greater in SHR than WKY cell extracts was provided by the phospho-MAPK immunoblots, which showed that the AII stimulated tyrosine phosphorylation of MAPK was greater in SHR than in WKY derived cells. This is in contrast to the detection of unphosphorylated MAPK proteins by immunoblot, which showed that the amounts of MAPK immunoreactive protein were the same in the two cell types, indicating that the enhanced MAPK activity is not due to increased expression of the kinase.

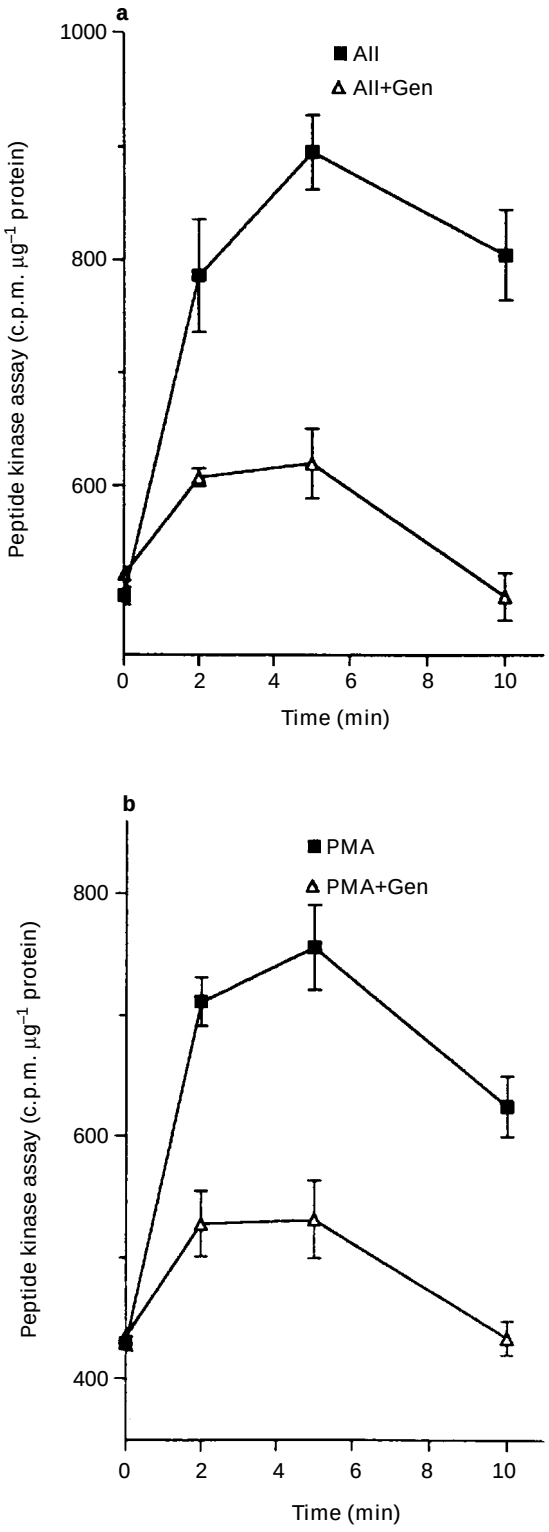

Figure 6 Effect of genistein (Gen) on the AII and PMA stimulated peptide kinase activity with SHR derived cells. AII (a) and PMA (b) were present at 100 nM for 5 min, with genistein 100 nM where indicated for a 20 min preincubation period as well as the stimulation period. Data are mean, with vertical lines indicating s.e.mean, from a single experiment carried out in triplicate and representative of 4 other experiments.

Our findings on MAPK activation by AII on these SHR and WKY cells are in contrast to those recently obtained by Lucchesi *et al*. (1996), who obtained similar peak levels of MAPK activation in both SHR and WKY following stimulation by AII, although MAPK was inactivated more rapidly in SHR cells. They also described an increased Ca^{2+} dependence of MAPK activation in SHR cells. However, in the present study, the main differences in MAPK activation by AII or PMA were in the peak responses, and inactivation rates were similar between SHR and WKY cells. Some of these differences may have been attributed to the cell types used, since Lucchesi *et al*. (1996) described no difference in the $[Ca^{2+}]_i$ response of their SHR and WKY cells, in contrast with results of ours (Baines *et al*., 1996) and others (Nabika *et al*., 1985; Bukoski, 1990; Bendhack *et al*., 1992).

The demonstration that the two cell types differ in their PLCδ content (Kato *et al*., 1992) provides a plausible explanation for these differences between SHR and WKY cells, if the enhanced responses in SHR cells are all downstream of an increased contribution of PLCδ in these cells. There are a variety of pathways proposed for the stimulation of MAPK by G protein-coupled receptors (e.g. Hawes *et al*., 1995), some of which are downstream of PLC. In investigating a possible difference between SHR and WKY derived cells with MAPK activity in the present study, it seemed likely therefore that any enhanced MAPK response to AII would be a consequence of enhanced activation of the PLC cascade. The results presented here show that this is not true. If the enhanced activation of MAPK by AII in SHR derived cells was solely a result of the greater stimulated activity of PLC, then it would not be expected that the stimulation of the protein kinase C pathway by PMA would result in a difference in MAPK activity between the cell types. The differential response of the two cell types with PMA stimulation, demonstrated here, implies an enhanced responsiveness of the pathway to MAPK which is independent of the previously described difference in PLC activity.

Activation of MAPK activity is known to require both tyrosine and threonine phosphorylations by the dual specificity kinase MEK (MAPK/extracellular signal related protein kinase). A variety of recent papers indicate complexity in the routes for AII stimulation of MAPK activities in vascular smooth muscle cells (e.g. Molloy *et al*., 1993; Leduc *et al*., 1995; Marrero *et al*., 1995; Scheiffer *et al*., 1996), including the possible involvement of tyrosine kinases upstream of MEK. We have investigated the involvement of a tyrosine kinase step in the MAPK cascade of SHR and WKY derived cells using both measurement of tyrosine phosphorylation by Western blots and inhibition of tyrosine kinases. The phosphotyrosine Western blots showed that there was widespread tyrosine phosphorylation in response to AII in both cell types, and that the response in the SHR cells was greater than in the WKY cells. PMA also stimulated tyrosine phosphorylation to a greater degree in SHR than WKY cells (Wilkie, N. & Boarder, M.R., unpublished observations). The results with the tyrosine kinase inhibitor genistein showed that both AII and PMA utilize a tyrosine kinase step in the pathway to activation of MAPK. Genistein did not inhibit the PLC response to AII in these cells, nor was it cytotoxic under the conditions used (Morton, C. & Boarder, M.R., unpublished observations). It is important for the interpretation of this work that results have

previously been published which show that genistein does not inhibit MEK (Winitz *et al*., 1993; van Biesen *et al*., 1995). The genistein sensitivity of PMA and AII stimulation of MAPK therefore indicates that there is another tyrosine phosphorylation step, upstream of the phosphorylation of MAPK. The tyrosine Western blots suggest that this step is greater in the SHR cells, although confirmation of this will await identification of the tyrosine kinase step and characterization in SHR and WKY cells. G protein-coupled receptor stimulated tyrosine kinase activities, both independent of PLC and downstream of PLC, have been described (van Biesen *et al*., 1995; Lev *et al*., 1995).

The genistein sensitivity of activation of MAPK by AII appears in conflict with the recent observations of Leduc *et al*. (1995), that AII stimulation of MAPK in cultured vascular smooth muscle cells is insensitive to genistein. While this may reflect a difference in the cell cultures used, it is worth noting that there are diverse results in the literature with respect to genistein sensitivity of heterotrimeric G protein-coupled MAPK activation, presumably a consequence of diverse pathways to MAPK.

We have provided evidence there that the response to AII is partially dependent on protein kinase C, with the demonstration that the early phase of the stimulation of both forms of MAPK activity was sensitive to Ro 31-8220 although the later phase of stimulation at 10 min was unaffected. An alternative explanation which cannot be excluded by the present data is that the effect of Ro 31-8220 was contributed to by effects independent of protein kinase C (Beltman *et al*., 1996); investigation of this hypothesis will require further studies with different PKC inhibitors. However, the central observation relating to protein kinase C in this paper derives from the PMA studies, which are independent of, yet consistent with, the Ro 31-8220 data.

Several conclusions arise from this study. Firstly, the stimulation of tyrosine phosphorylation by AII is greater in SHR cells than WKY. Secondly, the p42 and p44 forms of MAPK activity are more strongly stimulated in SHR cells than in WKY cells, in response to both AII and PMA. Thirdly, AII stimulation of both $p44^{mapk}$ and $p42^{mapk}$ activity in vascular smooth muscle cells involves an early phase which is protein kinase C-dependent, and a late phase which is independent of protein kinase C. Both phases are equally dependent on a tyrosine kinase step. Fourthly, PMA stimulates both $p44^{mapk}$ and $p42^{mapk}$ in vascular smooth muscle cells by a tyrosine kinase-dependent pathway. We propose as a model that AII stimulation of MAPK involves sequential protein kinase C and tyrosine kinase activation, and that there is upregulation of responses to AII stimulation in the SHR derived cells at the level of protein kinase C-mediated activation of tyrosine kinase. It is this enhanced tyrosine kinase activity which then leads to the increased MAPK response. Upregulation at this level is independent of the previously described upregulation of PLC responses. Further studies will be necessary to investigate the hypothesis that these differences in signalling at the MAPK level contribute to the hypertensive phenotype.

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