Angiotensin II causes phosphatidylinositol turnover and increases 1,2-diacylglycerol mass but is not mitogenic in rat liver T51B cells

Nicholas M. DEAN and Alton L. BOYNTON*

Basic Science Program, Cancer Research Center of Hawaii, 1236 Lauhala St., University of Hawaii, Honolulu, HI 96813, U.S.A.

Proliferation in rat liver T51B cells has previously been shown to be initiated by the tyrosine-kinase activator epidermal growth factor. We have found that T51B cells also contain angiotensin II receptors, and, as the transforming *mas* oncogene has been identified as a functional angiotensin receptor [Jackson, Blair, Marshall, Goedert & Hanley (1988) *Nature* (London) 335, 437–440], we have investigated the possibility that angiotensin II might also regulate proliferation of T51B cells. Angiotensin II at concentrations up to 10 μ M did not promote DNA synthesis, even in the presence of the co-mitogens serum (1 %) or 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (50 ng/ml). The addition of 1 μ M-angiotensin II to *myo*- 3 Hinositol-radiolabelled T51B cells did however result in a rapid accumulation of multiple inositol phosphates as well as in an increase in intracellular Ca^{2+} , demonstrating the coupling of the angiotensin receptor in these cells to a polyphosphoinositide-hydrolysing phospholipase C. The increases in both inositol phosphates and intracellular Ca^{2+} were lower in cells pretreated for 10 min with 50 ng of TPA/ml and potentiated by a 24 h pretreatment with TPA. In addition, angiotensin II increased 1,2-diacylglycerol levels. These results demonstrate that, although angiotensin II is capable of increasing phosphoinositide-derived second messengers in T51B cells, these responses are not sufficient to trigger DNA synthesis.

INTRODUCTION

The co-ordination of cell division in quiescent cells is regulated by the actions of various growth factors. Some of the better-characterized growth factor receptors contain intrinsic tyrosine-kinase activity, e.g. those for platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) (Yarden & Ullrich, 1988). A different class of receptors has been identified by genetic studies and shown to possess certain structural similarities, and furthermore do not appear to contain tyrosine-kinase activity. Examples include M-1 and M-3 muscarinic receptors (Bonner *et al.*, 1987), β -adrenergic receptors (Lefkowitz & Caron, 1988), 5-hydroxytryptamine receptors (Julius *et al.*, 1988) and the substance-K receptor (Masu *et al.*, 1987). This latter group of receptors also share certain biochemical responses upon binding of their respective agonists. These responses include activation of a phospholipase C (PLC) which hydrolyses PtdIns P_2 , and a subsequent elevation in intracellular Ca^{2+} ($[Ca^{2+}]_i$) caused by Ins(1,4,5) P_3 (see Berridge, 1987). Evidence is accumulating that, when activated, these receptors are also capable of eliciting a mitogenic response in cells which express them, for example substance-K receptors in smooth muscle cells (Nilsson *et al.*, 1985) and muscarinic receptors in Chinese hamster ovary cells transfected with different muscarinic receptor subtypes (Ashkenazi *et al.*, 1989). Receptors of this general structure have been further implicated in regulating the mitogenic response of cells in sequencing studies of the transforming *mas* oncogene, which have determined the structure of the gene and have identified it as another example of a receptor containing seven transmembrane domains (Young *et al.*, 1986). At that time, the endogenous ligand to the receptor was unknown; however, recent evidence has demonstrated that the *mas* oncogene is in fact a functional angiotensin receptor, and that cells stably transfected with the *mas* oncogene will respond mitogenically to

angiotensin (Jackson *et al.*, 1988). This raises the intriguing possibility that, in addition to its recognized roles in vascular functions and the regulation of electrolyte metabolism, angiotensin might function as a regulator of cell proliferation. The biochemical consequences of angiotensin II binding to its receptor have been studied in the former contexts, and the hormone has been shown to stimulate a PtdIns P_2 -specific PLC with the consequent release of Ins(1,4,5) P_3 and increased $[Ca^{2+}]_i$ (Kojima *et al.*, 1984; Johnson & Garrison, 1987; Baukal *et al.*, 1988), to activate an amiloride-sensitive Na^+/H^+ exchange (Berk *et al.*, 1987) and to induce *c-fos* mRNA (Taubman *et al.*, 1989).

However, it is the potential of angiotensin II to function as a regulator of cell proliferation that has interested us, in relation to the growth factor and Ca^{2+} requirements for specific cell-cycle transitions of quiescent T51B rat liver cells (Boynton *et al.*, 1989). Previous studies have demonstrated that quiescent T51B cells will undergo DNA synthesis in response to the tyrosine-kinase activator EGF in a manner that is dependent upon an influx of extracellular Ca^{2+} . This induction of the progression through the cell cycle does not appear to be initiated by factors whose mode of action is restricted to raising $[Ca^{2+}]_i$ from exclusively intracellular stores through increased PtdIns P_2 hydrolysis, e.g. thrombin and ATP (Hill *et al.*, 1988; Boynton *et al.*, 1989). We have found that T51B cells possess receptors for angiotensin II, and consequently we have determined the ability of this hormone to function as a mitogenic growth factor. In order to demonstrate a full biochemical response for the receptors, we have also investigated the changes in PtdIns turnover, 1,2-diacylglycerol (DAG) generation and Ca^{2+} signalling induced by angiotensin II. In addition, because of its effect as a co-mitogen in T51B cells, we have determined the effect of the protein kinase C (PKC) activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA) in regulating the generation of these signals.

Abbreviations used: BME, Eagle's basal medium with Earle's salts; $[Ca^{2+}]_i$, intracellular calcium concentration; DAG, 1,2-diacylglycerol; DETAPAC, diethylenetriaminepenta-acetic acid; EGF, epidermal growth factor; GroPtdIns, glycerophosphoinositol (similarly GroPtdIns P and GroPtdIns P_2 , with locants designating specific isomers where appropriate); PDGF, platelet-derived growth factor; PKC, protein kinase C; PLC, phospholipase C; PtdCho, phosphatidylcholine; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Cell culture

Rat liver T51B cells were plated at a density of $0.7 \times 10^4/\text{cm}^2$ in 100 mm plastic Petri dishes in 5 ml of Eagle's basal medium with Earle's salts (BME) containing 10% calf serum (Boynton *et al.*, 1985). The cultures were grown to confluence in this medium (usually 6–8 days after plating), at which time experiments were performed.

Measurement of DNA synthesis

The proportion of cells entering S-phase and undergoing DNA synthesis was determined by the uptake of [^3H]thymidine (2.5 $\mu\text{Ci}/\text{ml}$; New England Nuclear, Boston, MA, U.S.A.). Cells were then prepared for autoradiography and the fraction of cell nuclei containing the radioactive nucleoside was determined according to previously described methods (Boynton & Whitfield, 1976).

Measurement of [^3H]inositol phosphate generation

T51B cells were radiolabelled with *myo*-[^3H]inositol (14.6 Ci/mmol; New England Nuclear) at a concentration of 15 $\mu\text{Ci}/\text{ml}$ for 72 h before cell stimulation. After radiolabelling, the confluent 6–8 day cells were washed twice (5 min each) with 5 ml of BME (pH 7.4) buffered with 20 mM-Hepes and containing 10 mM-LiCl, and then a further 5 ml of the same medium was added (all at 37 °C). Angiotensin II (Sigma, St. Louis, MO, U.S.A.) was added to a final concentration of 1 μM . We also pretreated some cells with TPA (LC Service Corp., Woburn, MA, U.S.A.) (50 ng/ml) for either 10 min or 24 h before angiotensin II addition. At the required time after addition, medium was removed and the cells were killed with 250 μl of 5% trichloroacetic acid (containing 1 mg of phytic acid/ml) at 4 °C. The cells were scraped into this medium and the insoluble pellet and supernatant were collected by centrifugation (15600 *g*, 10 s) and processed to obtain the [^3H]inositol-radiolabelled phospholipids (see below) and the [^3H]inositol phosphates (Dean & Moyer, 1987).

The [^3H]inositol phosphates were resolved by h.p.l.c. using previously described techniques (Dean & Moyer, 1988; Dean & Beaven, 1989). Samples were separated on a Whatman Partisil 5 SAX RAC II column (10 cm $\times$ 0.47 cm) connected to a Beckman model 171 radioisotope detector (flow cell volume 1000 μl) with a buffer/scintillation fluid mixture of 1:4 (v/v) using Pico-flow IV scintillation fluid (Packard Instruments).

Measurements of [^3H]inositol phosphate lipid hydrolysis

The trichloroacetic acid-precipitated cell pellets from angiotensin II-treated [^3H]inositol-radiolabelled cells were extracted in 250 μl of chloroform/methanol/HCl (200:100:1, by vol.), and the supernatant was collected by centrifugation (15600 *g*, 10 s) and dried under N_2 . The lipids in this extract were deacylated in 400 μl of 25% methylamine/methanol/*n*-butanol (4:4:1, by vol.) at 56 °C for 45 min (Hawkins *et al.*, 1986). The sample was dried under N_2 , redissolved in 240 μl of 5% trichloroacetic acid and rapidly neutralized with water-saturated diethyl ether. The resulting [^3H]glycerophosphoinositols ([^3H]GroPtdIns) obtained were resolved by h.p.l.c. (general methods as for the [^3H]inositol phosphates) with an ammonium phosphate gradient as follows: isocratic 0.01 M (5 min), followed by 0.01–0.25 M (10 min) to elute GroPtdIns, 0.25–0.28 M (40 min) to elute GroPtdIns phosphate (GroPtdIns P) and 0.28–1.4 M (10 min), it was then held at 1.4 M for a further 25 min to elute GroPtdIns bisphosphate (GroPtdIns P_2), before being returned to 0.01 M. The gradient was characterized, and the [^3H]inositol-radiolabelled glycerophosphatidylinositols obtained from cells were identified by co-

chromatographing some samples with [^{32}P]GroPtdIns(4) P and [^{32}P]GroPtdIns(4,5) P_2 prepared from erythrocyte ghosts (Downes & Michell, 1981).

Measurements of [Ca^{2+}]_i

T51B cells were plated on round glass cover slips in 2 ml of BME containing 10% calf serum and used 6–8 days later when confluent. At this point the monolayers were loaded for 45 min with 7.5 μM -fura-2 AM (Molecular Probes, Eugene, OR, U.S.A.) and then washed once for 5 min in a Hepes-buffered salt solution (125 mM-NaCl, 6 mM-KCl, 1 mM-MgCl₂, 25 mM-Hepes and 5.5 mM-glucose, pH 7.4); this also contained either 1.8 mM-CaCl₂ (for + Ca^{2+} buffers) or 100 μM -EGTA (for – Ca^{2+} buffers). The coverslips were then placed in 2 ml of the same Hepes-buffered salt solution in disposable cuvettes, and the changes in fluorescence in response to angiotensin II (10 μM) were monitored at 340 nm excitation and 500 nm emission at 30 °C using a fluorescence spectrophotometer (Perkin-Elmer). The changes in [Ca^{2+}]_i were determined according to Grynkiewicz *et al.* (1984). In some experiments cells were pretreated with TPA (50 ng/ml) for either 10–20 min or 24 h before the addition of angiotensin II.

Measurement of DAG mass

Quiescent T51B cells were washed twice with 5 ml of BME (pH 7.4, buffered with 20 mM-Hepes) and angiotensin II (10 μM) added to the final wash. Incubations were terminated by aspirating the medium and adding 1 ml of ice-cold methanol to the cells (van Veldhoven & Bell, 1988). Cells were scraped into the methanol and the culture dishes washed with another 1 ml of methanol. The methanol extracts were pooled and added to 1 ml of chloroform, the organic phospholipid-containing layer was obtained by Bligh & Dyer extraction (Bligh & Dyer, 1959) and subsequently dried under N_2 , and the DAG content was determined using recombinant DAG kinase (Preiss *et al.*, 1986). This was accomplished by solubilizing the organic extract in 20 μl of 255 mM-*n*-octyl β -D-glucoside/5 mM-cardiolipin in 1 mM-diethylenetriaminepenta-acetic acid (DETAPAC), and then 50 μl of a reaction buffer (100 mM-imidazole, 100 mM-NaCl, 25 mM-MgCl₂ and 2 mM-EGTA), 5 μl of DAG kinase (Lipidex, Inc., Middleton, WI, U.S.A.) and 15 μl of freshly prepared 15 mM-dithiothreitol (in 1 mM-DETAPAC) were added. The reaction was started by the addition of 10 μl of 5 mM- γ - ^{32}P ATP (0.2 mCi/ μmol ; New England Nuclear) and terminated after 30 min at room temperature by the addition of 700 μl of 1 M-NaCl and then 3 ml of chloroform/methanol (1:2, v/v). The [^{32}P]phosphatidic acid generated was recovered from the organic phase, separated by t.l.c. on channelled silica-gel G plates (Analtech) using a chloroform/methanol/acetic acid (65:15:5, by vol.) mobile phase, and subjected to autoradiography. The spot corresponding to phosphatidic acid (R_f 0.8) was scraped and the radioactivity was determined by scintillation counting. The amount of DAG present in the sample was calculated based upon the specific activity of the ATP used for the kinase reaction (Preiss *et al.*, 1986).

RESULTS

DNA synthesis

Quiescent T51B cells treated with 20% serum responded with approx. 60–70% of cells replicating their DNA, whereas 50–60% of cells treated with 100 ng of EGF/ml in serum-free BME divided. The EGF response can be increased to levels similar to those obtained with 20% serum by the inclusion of 1% serum (itself not mitogenic) (Table 1). The addition of angiotensin II at concentrations up to 10 μM was not mitogenic, even in the

Table 1. Effects of serum, angiotensin II and EGF on DNA synthesis in quiescent G_0 -arrested T51B cells

The percentage of proliferating cells was determined by [3 H]-thymidine uptake and counting of radiolabelled nuclei as described in the Materials and methods section.

Addition	Proliferating cells (%)
None	< 1
Serum (20 %)	61.6 $\pm$ 3.9
Serum (1 %)	2.2 $\pm$ 0.8
Angiotensin II (10 μ M)	< 1
EGF (100 ng/ml)	50.7 $\pm$ 6.2
Angiotensin II (10 μ M) + 1 % serum	2.0 $\pm$ 0.9
EGF (100 ng/ml) + 1 % serum	67.2 $\pm$ 3.4

presence of 1 % serum (Table 1) or in cells pretreated with 50 ng of TPA/ml (results not shown), both of which potentiate the EGF-induced mitogenic response in T51B cells (Hill *et al.*, 1988).

Inositol phosphate generation

myo-[3 H]inositol-radiolabelled T51B cells contained multiple isomers of inositol phosphates which could be readily separated by h.p.l.c. The addition of 1 μ M-angiotensin II to T51B cells in the presence of 1.8 mM extracellular Ca^{2+} resulted in the rapid (within 10 s) accumulation of $Ins(1,4,5)P_3$, $InsP_4$ and $Ins(1,4)P_2$ (Figs. 1a–1c). $Ins(1,4,5)P_3$ levels increased by 50 % after 30 s of stimulation and returned to control levels after only 1 min. By contrast, the increase in $InsP_4$ was biphasic, with the concentration doubling after 15 s of stimulation, falling to near control levels after 1 min and increasing again after 15 min. Levels of $Ins(1,4)P_2$ increased by almost 3-fold by 30 s of angiotensin treatment before declining to control levels after 2 min (Fig. 1a). By 1 min of stimulation, levels of $Ins(1,3,4)P_3$ had also risen 3-fold (Fig. 1b), and the concentration of $Ins(4)P$ had risen 10-fold (Fig. 1a), but the concentration of $Ins(1)P$ did not increase until after approx. 2–3 min of stimulation. Levels of $Ins(1,3)P_2$ were undetectable (< 300 c.p.m./peak) in resting cells; however, by 5 min of stimulation they had reached 1000 c.p.m. before falling to control levels after 15 min (Fig. 1c). Two isomers of $InsP_5$ and $InsP_6$ were also present, but the levels of these were not changed by 15 min of treatment with angiotensin II.

PKC activation by phorbol esters has been shown to inhibit the generation of inositol phosphates in many cell types (Brown *et al.*, 1987). In T51B cells, TPA has a potentiating effect on EGF-induced DNA synthesis (Hill *et al.*, 1988). Although we have found that TPA does not affect the lack of a mitogenic response of T51B cells to angiotensin, we have determined whether phorbol esters can affect the angiotensin II-stimulated hydrolysis of $PtdInsP_2$. Pretreating [3 H]inositol-radiolabelled T51B cells for 10 min with 50 ng of TPA/ml before the addition of hormone resulted in a decreased accumulation of all inositol phosphates compared with control levels; for example, $Ins(4)P$ is shown in Fig. 2. Such an inhibition has previously been reported for the angiotensin II-induced inositol phosphate accumulation in smooth muscle cells and adrenal glomerulosa cells (Griendling *et al.*, 1986; Woodcock *et al.*, 1988).

We have also investigated the effects of down-regulating PKC on inositol phosphate turnover, as this down-regulation has been shown to elevate both basal and mitogen (bombesin)-stimulated inositol phosphate production in 3T3 cells (Brown *et al.*, 1987).

Fig. 1. Accumulation of [3 H]inositol phosphates in [3 H]inositol-radiolabelled quiescent G_0 -arrested T51B cells in response to 1 μ M-angiotensin II

T51B cells were radiolabelled with 15 μ Ci of [3 H]inositol/ml and stimulated with 1 μ M-angiotensin II, and the inositol phosphate isomer levels were determined by h.p.l.c. using the protocols described in the Materials and methods section. Each point represents the mean of two separate samples in a single typical experiment which was repeated four times with similar results. (a) ○, $Ins(1)P$; ●, $Ins(4)P$; △, $Ins(1,4)P_2$; (b) ○, $Ins(1,4,5)P_3$; ●, $Ins(1,3,4)P_3$; (c) ○, $InsP_4$; ●, $Ins(1,3)P_2$.

The preincubation of cells with TPA for 24 h removes PKC activity by causing a persistent association of the enzyme with cellular membranes, followed by proteolytic degradation (Nishizuka, 1988). We have found that such a preincubation with TPA removes > 95 % of PKC activity from both cytosol and membrane fractions of T51B cells (results not shown). Basal levels of inositol and isomers of inositol phosphates were unchanged in cells with down-regulated PKC; however, when angiotensin II was added to these cells, the turnover of inositol phosphates was greater than in non-TPA-treated cells. In a typical experiment of this type, in cells treated with TPA for 24 h and then with 1 μ M-angiotensin II for 5 min, $Ins(4)P$ levels were 39860 ± 1505 c.p.m. (mean $\pm$ S.D.), whereas in cells not treated with TPA, angiotensin II increased $Ins(4)P$ levels to 23408 ± 1429 c.p.m. after 5 min (Fig. 2).

Phosphoinositide hydrolysis

Phosphatidylinositol lipids phosphorylated at the D-3 position around the inositol ring have been described which have been implicated in the control of cell proliferation (Whitman *et al.*, 1988). Consequently we have examined *myo*-[³H]inositol-radiolabelled T51B cells for the presence of these PtdIns lipids with phosphates in positions other than the conventional 1, 4 and 5 positions. When deacylated [³H]inositol-radiolabelled phospholipids were analysed by h.p.l.c., we have found, in addition to the expected peaks of radioactivity corresponding to GroPtdIns(4)*P* and GroPtdIns(4,5)*P*₂ (with retention times of 55–58 min and 74–77 min respectively), a peak of radioactivity eluting 2–3 min ahead of GroPtdIns(4)*P*. This peak contained approx. 10–12% of the radioactivity present in GroPtdIns(4)*P*, and, by comparison with previously published data (Whitman *et al.*, 1988; Lips *et al.*, 1989) is tentatively identified as GroPtdIns(3)*P* derived from PtdIns(3)*P*. The addition of angiotensin

II to [³H]inositol-radiolabelled cells resulted in a transient decrease of 15–20% in levels of GroPtdIns(4)*P* and GroPtdIns(4,5)*P*₂; however, levels of GroPtdIns(3)*P* remained unchanged (results not shown). The amounts of all lipids had returned to control levels after 5 min of stimulation, indicating the rapid recycling and phosphorylation of PtdIns.

Measurement of Ca²⁺

The addition of 1 μM-angiotensin II to fura-2-loaded T51B cells in the presence of 1.8 mM external Ca²⁺ produced a rapid transient spike of [Ca²⁺]_i which lasted for 1 min, after which time the [Ca²⁺]_i fell to approx. one-third of maximal levels. This was followed by a more sustained elevation in [Ca²⁺]_i which lasted for up to 10 min after hormone addition (Fig. 3a). In the presence of low (0.025 mM) external calcium, [Ca²⁺]_i increased with angiotensin II treatment; however, the magnitude of the response was generally smaller and the peak was shorter in duration. In addition, the sustained phase of elevation was not seen, with the [Ca²⁺]_i returning to near control levels within 1 min of hormone addition (Fig. 3b).

Fig. 2. Accumulation of [³H]Ins(4)*P* in [³H]inositol-radiolabelled TPA-treated T51B cells in response to 1 μM-angiotensin II

T51B cells were radiolabelled with 15 μCi of [³H]inositol/ml, treated with 50 ng of TPA/ml for either 10 min or 24 h and then stimulated with 1 μM-angiotensin II. Ins(4)*P* levels were determined as described in the Materials and methods section. Each point represents the mean of three separate samples in a single typical experiment. ○, +TPA (10 min); △, +TPA (24 h); □, control (no TPA addition).

Fig. 4. Accumulation of DAG in quiescent G₀-arrested T51B cells in response to 1 μM-angiotensin II

Quiescent T51B cells were stimulated with 1 μM-angiotensin II and at the required time extracts were prepared and the DAG mass was determined as described in the Materials and methods section. Each point is the mean of eight or nine values (except 10 min control; *n* = 6) (± S.E.M.) obtained in three separate experiments and are for the DAG content of a 100 mm dish of cells. ○, +Angiotensin II (1 μM); ●, control.

Fig. 3. Changes in [Ca²⁺]_i in T51B cells in response to 1 μM-angiotensin II

Quiescent T51B cells were loaded with 7.5 μM-fura-2 AM and stimulated with 1 μM-angiotensin II, and the changes in fluorescence were monitored as described in the Materials and methods section. The arrows indicate the addition of angiotensin II. Experiments were performed in (a) Ca²⁺-containing (1.8 mM-CaCl₂) buffer; (b) Ca²⁺-free (+ 100 μM-EGTA) buffer; (c) Ca²⁺-containing buffer in cells pretreated with 50 ng of TPA/ml for 24 h.

A 10 min pretreatment of T51B cells with 50 ng of TPA/ml in the presence of 1.8 mM external calcium greatly diminished, or abolished altogether, the rise in $[Ca^{2+}]_i$ obtained with angiotensin II (results not shown). The preincubation of T51B cells for 24 h with 50 ng of TPA/ml enhanced the $[Ca^{2+}]_i$ signal obtained with angiotensin II in the presence of 1.8 mM external calcium. The calcium increase returned to plateau levels more slowly than in control (untreated) cells, and remained elevated above basal levels for a longer period of time (Fig. 3c).

Production of DAG

To determine whether levels of DAG are transiently elevated or remain high for more prolonged periods during angiotensin II stimulation of T51B cells, DAG levels were measured using a protocol for quantifying changes in DAG mass. DAG levels in a 100 mm diam. dish for quiescent non-stimulated cells were 872 ± 50 pmol ($n = 9$) (mean $\pm$ S.E.M.), and were increased to 1067 ± 78 pmol ($n = 8$) by 30 s of stimulation with angiotensin II. Levels of DAG were maximally elevated at 2.5 min, reaching 1482 ± 88 pmol ($n = 9$), and, although somewhat diminished, were still increased above control levels at 10 min after the addition of angiotensin II (Fig. 4), at 1369 ± 120 pmol.

DISCUSSION

The results presented here demonstrate that in rat liver T51B cells, angiotensin receptors are coupled to a PtdIns P_2 -hydrolysing PLC, and cause a large increase in Ins(1,4,5) P_3 , DAG and $[Ca^{2+}]_i$ when activated. These results confirm observations obtained in cultured vascular smooth muscle cells (Brock *et al.*, 1985), adrenal glomerulosa cells (Kojima *et al.*, 1984; Balla *et al.*, 1986) and cultured hepatocytes (Lynch *et al.*, 1985; Bockkino *et al.*, 1985). Angiotensin II addition also transiently decreased levels of PtdIns(4) P and PtdIns(4,5) P_2 in T51B cells; however, levels of the minor lipid PtdIns(3) P remain unchanged. In addition, we have been unable to detect an immediate increase in the product of PLC-mediated hydrolysis of this lipid, namely Ins(1,3) P_2 . Ins(1,3) P_2 does increase after 2–3 min, but this may be the result of Ins(1,3,4) P_3 dephosphorylation. PtdIns(3) P has been shown to be a poor substrate *in vitro* for bovine brain PLC II (Lips *et al.*, 1989), which may explain the apparent lack of effect of angiotensin on levels of this lipid. Whether the inability of angiotensin II to affect PtdIns(3) P levels is related to its lack of mitogenic activity remains to be determined.

Phorbol esters have been shown previously to exert a potentiating effect on the EGF-induced mitogenic response of T51B cells (Hill *et al.*, 1988) and in Swiss 3T3 cells responding to either insulin or EGF (Hesketh *et al.*, 1988). TPA also potentially affected the generation of the angiotensin II-induced inositol phosphate accumulation and the Ca^{2+} signal, but with contrasting results. Short-term preincubation of T51B cells with TPA inhibits both PtdIns P_2 hydrolysis and Ca^{2+} signalling, whereas a 24 h TPA pretreatment has a potentiation effect. A number of possible sites at which PKC might be mediating its effect on PLC activation have been suggested (Brown *et al.*, 1987; Nishizuka, 1988); however, regardless of the mechanism involved, it appears that PKC is involved in the normal regulation of an angiotensin II-activated PLC in a manner which suggests a negative-feedback mechanism of the type proposed by Nishizuka (1988). TPA pretreatments of T51B cells are however without any potentiating effect on the stimulation of DNA synthesis by angiotensin II.

Long-term cellular responses to hormones, e.g. cell proliferation, have been suggested to be regulated by the association of PKC with the plasma membrane in a manner which is regulated by an interplay between $[Ca^{2+}]_i$ and DAG (Alkon & Rasmussen, 1988). As we have shown, angiotensin II produces a large

increase in $[Ca^{2+}]_i$, but we have been unable to find a shift in PKC activity from the cytosol to the membrane in response to this hormone (results not shown). Consequently we considered the possibility that DAG production might be deficient in these cells, resulting in a non-proliferative response to angiotensin II. However, DAG levels exhibited a 2-fold increase and remained elevated for up to 10 min after angiotensin II addition, even after inositol phosphate generation appeared to be desensitized. Some evidence suggests that DAG production might also arise from sources other than PtdIns and that the DAG derived from PtdIns might represent only a small percentage of the total DAG generated in response to hormone stimulation (Kennerly, 1987). One alternative source is phosphatidylcholine (PtdCho), which might yield DAG directly, through PLC-mediated hydrolysis, or indirectly by the action of a phosphatidic acid phosphatase subsequent to the hydrolysis of PtdCho by a phospholipase D (Cockcroft, 1984; Exton, 1988; Pelech & Vance, 1989). However, by measuring changes in both intracellular and extracellular $[^3H]$ choline-radiolabelled metabolites in T51B cells, we have found that PtdCho hydrolysis is not affected by the addition of angiotensin II (results not shown). Therefore alternative sources to both PtdIns and PtdCho may be providing DAG (Kennerly, 1987).

The question remains as to why angiotensin II is mitogenic in cells transfected with the *mas* oncogene but not in T51B cells with functioning angiotensin II receptors, and a number of different possibilities are evident. (1) The *mas* oncogene may not be the same receptor as is present in T51B cells, and could represent either a different sub-type or a mutated version of the angiotensin receptor generating different or amplified intracellular signals. (2) Although identical, the receptors may produce different signals which are dictated by other cellular factors (for example the presence of a type I PtdIns kinase) which would consequently yield different cellular responses. (3) The intracellular signals generated by the two receptors are identical, but different cells may be preprogrammed to respond differently to the same set of signals, a response which might be affected by the function or state of differentiation of the cell. Which of these possibilities is most likely remains to be determined, as does the role of angiotensin in regulating cell proliferation *in vivo*. Our results do however demonstrate that the coupling of a hormone to a PtdIns P_2 -hydrolysing PLC is not necessarily sufficient to evoke a mitogenic response in a cell expressing that receptor, and that other signal-transducing pathways may be required to promote DNA synthesis.

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