Biochem. J. (1992) 285, 759–766 (Printed in Great Britain)

Rapid desensitization of vasopressin-stimulated phosphatidylinositol 4,5-bisphosphate and phosphatidylcholine hydrolysis questions the role of these pathways in sustained diacylglycerol formation in A10 vascular-smooth-muscle cells

Robin PLEVIN* and Michael J. O. WAKELAM†
Molecular Pharmacology Group, Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

The kinetics of vasopressin-stimulated PtdIns(4,5)P_2 and phosphatidylcholine (PtdCho) hydrolysis in relation to sustained diacylglycerol (DAG) formation was investigated in A10 vascular-smooth-muscle cells in culture. Vasopressin stimulated a transient increase in Ins(1,4,5)P_3 mass formation, which was mirrored by a decrease in PtdIns(4,5)P_2 mass levels. Vasopressin stimulated sustained accumulation of total [^{3}H]inositol phosphates ([^{3}H]IP) in the presence of Li^+; however, this was significantly decreased by adding a vasopressin-receptor antagonist at different times after initial stimulation. Vasopressin-stimulated phospholipase D (PLD) activity was found to be a transient phenomenon lasting approx. 2 min. Experiments involving agonist preincubation with subsequent addition of butanol confirmed that vasopressin-stimulated PLD activity was desensitized. Vasopressin stimulated an increase in formation of choline, but not of phosphocholine, suggesting that PLD was the major catalytic route of PtdCho hydrolysis in this cell line. The roles of choline and inositol phospholipid hydrolysis in the prolonged phase of DAG formation was examined by comparing vasopressin-stimulated changes in DAG levels in the presence of butanol, the protein kinase C inhibitor Ro-31-8220 or a V_{1a}-receptor antagonist. Vasopressin-stimulated DAG formation was decreased by 40–50% in the presence of butanol between 1 and 10 min; however, during more prolonged stimulation butanol was without significant effect. In cells pretreated with Ro-31-8220, vasopressin-stimulated DAG formation was decreased by approx. 30% at 2 min, but was significantly potentiated at later times. This coincided with an enhancement of vasopressin-stimulated [^{3}H]IP accumulation. In cells exposed to the V_{1a}-receptor antagonist 5 min after addition of vasopressin, subsequent DAG formation was significantly decreased, indicating that sustained formation of DAG, like [^{3}H]IP accumulation, was dependent on continual agonist receptor activation. The results are discussed in terms of different phospholipid-hydrolytic pathways providing DAG generation.

INTRODUCTION

It is well established that a number of vasoconstrictors, such as angiotensin II (Griendling *et al.*, 1986) and endothelin-1 (Sunako *et al.*, 1990), stimulate the biphasic formation of the second messenger *sn*-1,2-diacylglycerol (DAG) in vascular-smooth-muscle cells in culture (VSMCC). This has been ascribed to be the result of two inter-related processes. Agonist receptor occupation results in the rapid hydrolysis of PtdIns(4,5)P_2 which releases simultaneously Ins(1,4,5)P_3 and DAG. However, initial formation of DAG from this source is believed to be short-lived, owing to desensitization of PtdIns(4,5)P_2 hydrolysis (Cook *et al.*, 1990; Palmer *et al.*, 1991) and the activity of DAG kinase (Bishop *et al.*, 1986; Ford & Gross, 1990). The second, more prolonged, phase is thought to be due to the sustained hydrolysis of another phospholipid, phosphatidylcholine (PtdCho). This may be achieved through two distinct catalytic pathways: direct hydrolysis of PtdCho by a PtdCho-specific phospholipase C (PLC), or phospholipase D (PLD)-activated hydrolysis followed by the conversion of the initial product, phosphatidic acid, into DAG by the action of phosphatidate phosphohydrolase (see review by Billah & Anthes, 1990). Since DAG is the physiological activator of protein kinase C (PKC) (Nishizuka, 1984), regulation of its formation may have important implications for the contractile process. Recently, it has been suggested that phosphatidic acid itself, in addition to providing a source of sustained DAG, may also have a role in the initiation of agonist-stimulated vascular smooth-muscle contraction (Ohanian *et al.*, 1990).

In a previous report we have shown that vasopressin, acting via a V_{1a} receptor, stimulates PLD-catalysed PtdCho breakdown in A10 VSMCC (Plevin *et al.*, 1991*b*). This process is kinetically downstream of initial PtdIns(4,5)P_2 hydrolysis and involves the intermediate activation of PKC. We therefore sought to determine how both pathways may contribute to the sustained formation of DAG in this cell line. The results in this present study suggest that, whereas vasopressin-stimulated PtdIns(4,5)P_2 and PtdCho hydrolyses are seemingly transient events and rapidly desensitized, formation of DAG is sustained. At later time points DAG is apparently derived predominantly from sources other than PtdIns(4,5)P_2 or PtdCho.

METHODS

A10 rat vascular smooth-muscle cells were maintained in Dulbecco's modified Eagle's medium containing 15% (v/v) foetal-calf serum at 37 °C in a humidified atmosphere of air/CO_2

Abbreviations used: IP, total inositol phosphates; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PLD, phospholipase D; DAG, *sn*-1,2-diacylglycerol; PLC, phospholipase C; PtdBuOH, phosphatidylbutanol; PtdCho, phosphatidylcholine; VSMCC, vascular smooth muscle cells in culture.

* Present address: Department of Physiology and Pharmacology, University of Strathclyde, Glasgow G1 1XW, Scotland, U.K.
† To whom correspondence should be addressed.

(19:1). For experiments, cells were seeded in 24-well plates and labelled for 72 h with [^{3}H]palmitic acid (1 μCi/ml) or [*methyl*-^{3}H]choline chloride (5 μCi/ml) in the Dulbecco's medium containing 2% foetal-calf serum.

On the day of the experiments, the labelling medium was removed and the cells were washed with 2 × 0.5 ml of Hanks buffered saline, pH 7.4, containing 1% (w/v) BSA and 10 mM-glucose. For the measurement of intracellular choline formation, the cells were incubated with agonist for the times indicated, the medium was aspirated and the reaction was terminated by addition of 0.5 ml of ice-cold methanol. For total choline estimation, the medium was aspirated before methanol addition. For assay of phosphatidylbutanol (PtdBuOH) accumulation, [^{3}H]palmitic acid-labelled cells were preincubated with 30 mM-butanol for 5 min before initiation of the reaction. The reaction was terminated by rapid aspiration, followed by addition of 0.5 ml of ice-cold methanol.

For measurement of choline metabolites, the cells were scraped, washed (0.25 ml of methanol) and the extracts transferred to insert vials. Chloroform (0.3 ml) was added and the samples were allowed to extract at room temperature (30 min). The phases were split by addition of chloroform (0.5 ml) and water (0.5 ml), followed by centrifugation (1000 *g*-min). A portion of the upper aqueous phase was analysed for glycerophosphocholine, phosphocholine and choline by cation-exchange chromatography on Dowex-50W (H^+) columns (Cook & Wakelam, 1989). For measurement of PtdBuOH formation the cell extracts were treated as above, except that glass vials were used throughout. The aqueous phase was removed and the lower phase dried down under vacuum. The sample was redissolved in chloroform/methanol (19:1, v/v) and applied to Whatman LK5DF plates as described by Cook *et al.* (1991) and developed with the upper phase of 2,2,4-trimethylpentane/ethyl acetate/acetic acid/water (5:11:2:10, by vol.). The location of the peak was established by co-migration with an external [^{3}H]PtdBuOH standard (R_F 0.4). Preliminary experiments confirmed the dose-dependency of [^{3}H]PtdBuOH formation on butan-1-ol concentration (results not shown).

For Ins(1,4,5)P_3 mass measurements, unlabelled cells were grown to confluency on 6 cm-diameter dishes. Cells were incubated with agonist in a final volume of 300 μl for the times indicated, and the reaction was terminated by addition of 25 μl of 20% (v/v) $HClO_4$. The cells were scraped, transferred to vials and the extracts neutralized with 1 M-KOH/60 mM-Hepes. A sample of the neutralized extract (200 μl) was assayed for Ins(1,4,5)P_3 by the method of Palmer *et al.* (1989) by using competitive displacement of [^{3}H]Ins(1,4,5)P_3 binding to adrenal-cortex microsomes quantified by using a standard curve.

Mass levels of PtdIns(4,5)P_2 were assayed as described by Palmer *et al.* (1991). Cells on 3 cm-diameter dishes were incubated with agonist in a final volume of 300 μl as outlined above. After termination of the reaction with 300 μl of ice-cold 20% (v/v) trichloroacetic acid, the extracts were washed once with 500 μl of 5% trichloroacetic acid/1 mM-EDTA and then once with 500 μl of water. The inositol-containing phospholipids were extracted for 60 min in 500 μl of chloroform/methanol/HCl (200:100:1, by vol.) at room temperature. After addition of chloroform (165 μl) and 0.1 M-HCl (300 μl), the phases were separated by centrifugation and a sample of the organic phase was dried under vacuum. The sample was re-dissolved in 250 μl of 1 M-KOH and incubated at 100 °C for 30 min. The samples were then passed through a 250 μl Dowex column (H^+; 200–400 mesh) and washed with 3 × 0.75 ml of water. The eluate was washed (3 × 3 ml) with light petroleum (b.p. 60–80 °C)/butanol (5:1, v/v), and a sample of the lower phase (250 μl) was freeze-dried and assayed for PtdIns(4,5)P_2 mass as Ins(1,4,5)P_3 as outlined above.

DAG mass was measured as described by Paterson *et al.* (1991). Briefly, cells in 6 cm-diameter dishes were incubated with agonist in a final volume of 750 μl, and the reaction was terminated by rapid aspiration followed by addition of 500 μl of ice-cold methanol. The cells were then treated as outlined above for the PtdBuOH assay. A sample of the lower chloroform layer was either used immediately for the DAG kinase reaction or stored overnight under N_2 at −20 °C. Dried samples or *sn*-1-stearoyl-2-arachidonylglycerol standards (25–1000 pmol) were incubated with DAG kinase and [γ-^{32}P]ATP in a mixed micelle preparation [6 mol% phosphatidylserine/0.3% (w/v) Triton X-100] in 100 mM-imidazole buffer (pH 6.6). The products were separated by t.l.c. with a solvent of chloroform/methanol/acetic acid (26:6:3, by vol.) and the labelled phosphatidic acid was excised from the plate and counted for radioactivity. DAG mass was quantified by using the *sn*-1-stearoyl-2-arachidonylglycerol standard curve. Recoveries in the range of 80–90% were linear over the standard curve and not affected by tissue extract (see Paterson *et al.*, 1991).

All radiolabelled compounds were obtained from Amersham International (Amersham, Bucks., U.K.). All other compounds were of the highest grades commercially available from previously reported sources. Ro-31-8220 was kindly given by Roche Products.

RESULTS

The kinetics of vasopressin-stimulated changes in Ins(1,4,5)P_3 mass are shown in Fig. 1. Second-messenger mass increased rapidly in response to 30 nM-vasopressin, reaching a peak between 10 and 20 s at approx. 6–7 times control values (Fig. 1*a*). Stimulated levels then declined rapidly, returning to basal between 2 and 5 min. Further challenge with excess peptide (100 μM) after 5 min or after intermediate incubation with 300 nM of the V_{1a}-receptor antagonist des-Gly^9-[β-mercapto-$\beta\beta$-cyclopentamethylenepropionyl-OEt-Tyr^2,Val^4,Arg^8]vasopressin failed to elicit a second increase in Ins(1,4,5)P_3, suggesting that PtdIns(4,5)P_2 hydrolysis was desensitized. PtdIns(4,5)P_2 levels were also decreased in response to vasopressin stimulation (Fig. 1*b*), falling to 65% of basal values within 15 s (control, 22.8 ± 5.8 pmol/sample; vasopressin, 15.30 ± 3.30 pmol/sample). Stimulated values returned to basal generally after 5 min and always within 10 min (control, 28.8 ± 2.40 pmol/sample; vasopressin, 29.00 ± 3.30 pmol/sample). The time course of vasopressin-stimulated accumulation of total [^{3}H]inositol phosphates [^{3}H]IP is shown in Fig. 2. After an initial rapid phase between 0 and 5 min, accumulation proceeded at a slower rate from 5 to 45 min. However, this decreased rate of vasopressin-stimulated accumulation of [^{3}H]IP was significantly further decreased by addition of excess V_{1a}-receptor antagonist. Addition of antagonist at 10, 15 and 20 but not at 30 min also decreased the accumulation of [^{3}H]IP at 45 min (results not shown).

The kinetics of PLD-activated PtdCho hydrolysis was examined by assessing the formation of PtdBuOH in cells labelled with palmitic acid and preincubated with 30 mM-butanol (Fig. 3). After a lag of some 10 s, vasopressin stimulated a rapid increase in the accumulation of PtdBuOH between 15 and 60 s (Fig. 3*a*). However, the rate of accumulation then slowed up to 2–3 min, after which no further PtdBuOH generation was observed. This contrasted with phorbol 12-myristate 13-acetate (PMA)-stimulated PtdBuOH accumulation, which, after a lag time of some 30 s, continued unabated for up to 15 min (Fig. 3*b*), suggesting that the desensitization of PLD activation was agonist-related. Steady-state accumulation of [^{3}H]PtdBuOH was not affected by addition of the V_{1a}-receptor antagonist, confirming

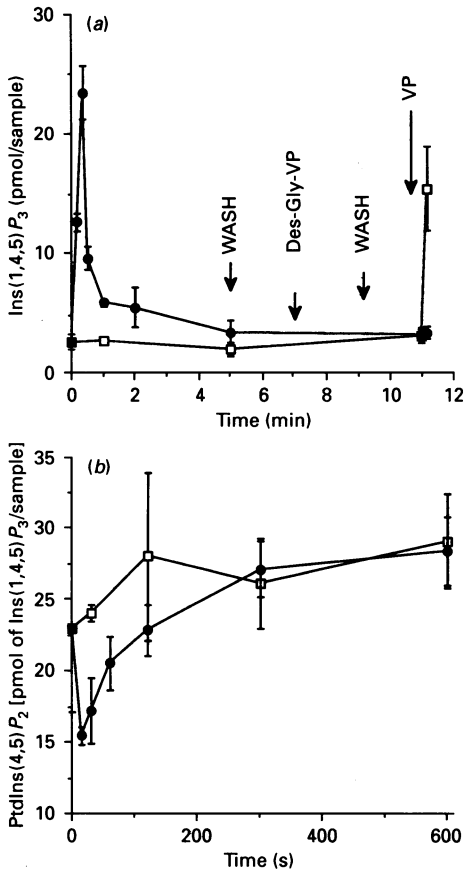

Fig. 1. Apparent desensitization of vasopressin-stimulated Ins(1,4,5)P_3 formation (*a*) and PtdIns(4,5)P_2 hydrolysis (*b*) in A10 VSMCC

In (*a*) cells were stimulated with vasopressin (30 nM) (●) or buffer (□) for the times indicated for 5 min, washed twice with buffer for 2 min, incubated with 300 mM-des-Gly^9-[β-mercapto-$\beta\beta$-cyclopentamethylenepropionyl-OEt-Tyr^2,Val^4,Arg^8]vasopressin (Des-Gly-VP) for 2 min, washed twice with buffer (2 min), and then challenged with vasopressin (VP; 100 μM) for 10 s, and Ins(1,4,5)P_3 mass was determined as outlined in the Methods. Control values (i.e. buffer pretreatment, buffer stimulation and vasopressin pretreatment, or buffer stimulation) were not significantly different from each other and are omitted for sake of clarity. In (*b*) cells were stimulated with 100 nM-vasopressin (●) or vehicle (□) for the times indicated. PtdIns(4,5)P_2 mass was determined as outlined in the Methods section. Each point represents the mean ± S.D. of triplicate determinations from a single representative experiment where $n = 3$.

the short duration of the agonist-dependent signal (results not shown).

The possibility that agonist-stimulated PLD activity was desensitized was further examined (Table 1). Pretreatment with vehicle for 5 min and then co-incubation with butanol plus vasopressin (100 nM) or PMA (100 nM) elicited a maximum PtdBuOH response as outlined above. However, preincubation with 100 nM-vasopressin in the absence of butanol for 5 min resulted in the complete abolition of PtdBuOH accumulation induced by a subsequent addition of either vehicle or vasopressin plus butanol. PMA, in the presence of butanol, added after preincubation with vasopressin still stimulated the accumulation of PtdBuOH formation; however, the response was decreased by 40% in comparison with that obtained after preincubation with vehicle (Table 1).

In order to confirm that the desensitization effect was not a result of an artifact of the transphosphatidylation assay or of possible toxic effects of butanol preincubation, the kinetics of

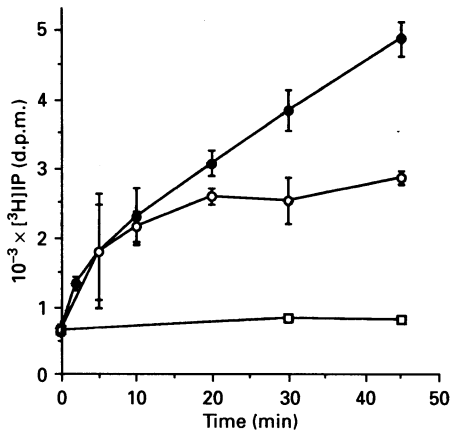

Fig. 2. Effect of V_{1a}-receptor antagonist on the kinetics of vasopressin-stimulated [^{3}H]IP accumulation in A10 VSMCC

Cells, prelabelled with 1 μCi of [^{3}H]inositol/ml for 72 h, were incubated with 20 mM-vasopressin (○, ●), or buffer (□) for the times indicated, in the presence (○) or absence (●) of the V_{1a} antagonist added after 5 min. Total [^{3}H]IP accumulation was measured as outlined in the Methods section. Each point represents the mean ± S.D. of triplicate determinations from a single experiment representative of four.

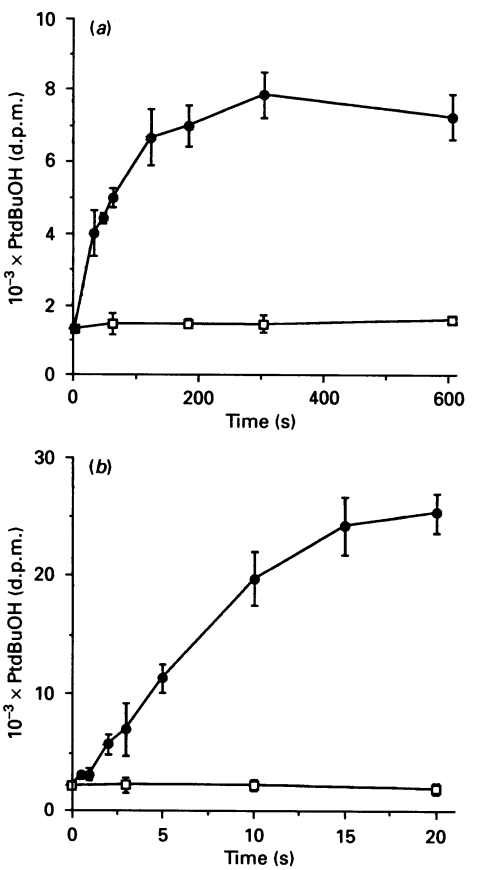

Fig. 3. Time course of vasopressin- and PMA-stimulated PtdBuOH accumulation in A10 VSMCC

Cells prelabelled with [^{3}H]palmitate were preincubated with 30 mM-butanol for 5 min and then stimulated with vehicle (□), vasopressin (●) (panel *a*) or PMA (●) (panel *b*) for the times indicated. PtdBuOH formation was determined as outlined in the Methods section. Each point represents the mean ± S.D. of triplicate determinations from a single experiment representative of three others (i.e. $n = 4$).

Table 1. Effect of vasopressin pretreatment on vasopressin- and PMA-stimulated PtdBuOH accumulation in A10 VSMCC

Cells prelabelled with [^{3}H]palmitate were preincubated with buffer or 100 nM-vasopressin for 5 min, then re-challenged with vehicle (control), vasopressin (100 nM) or PMA (100 nM) in the presence of 30 mM-butanol for a further (A) 2 or (B) 5 min. Each value represents the mean ± S.D. of triplicate determinations from a single experiment where $n = 3$ (expressed in d.p.m.).

		[^{3}H]PtdBuOH (d.p.m.)	
	Pretreatment (5 min)...	Buffer	Vasopressin (100 nM)
A	Control	1853 ± 246	1554 ± 280
	Vasopressin	9865 ± 1124	2490 ± 195
	PMA	6225 ± 260	4587 ± 511
B	Control	1381 ± 138	1451 ± 267
	Vasopressin	9545 ± 848	1577 ± 199
	PMA	12873 ± 1200	8208 ± 855

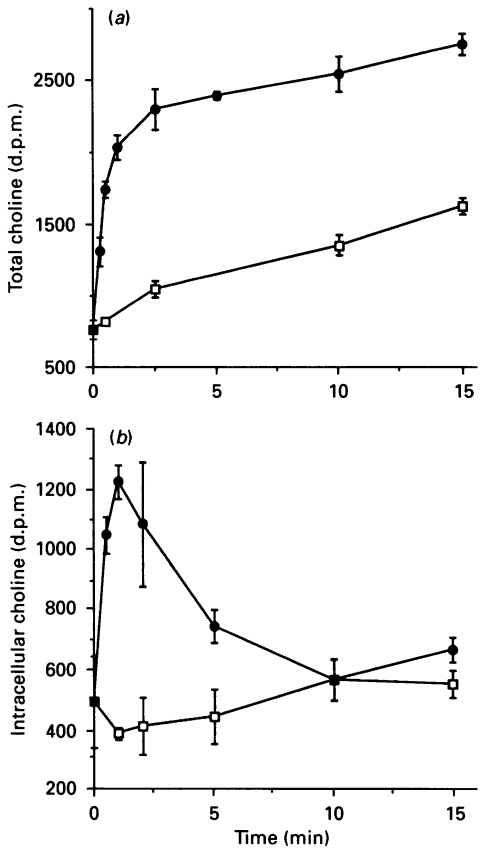

Fig. 4. Time course of vasopressin-stimulated (*a*) total and (*b*) intracellular choline formation in A10 VSMCC

Cells, prelabelled with [^{3}H]choline, were stimulated with vehicle (□) or 100 nM-vasopressin (●) for the times indicated. Water-soluble choline metabolites were analysed as outlined in the Methods section. Each point represents the mean ± S.D. of triplicate determinations from a single typical experiment where $n = 3$.

labelled choline formation was also assessed in response to both vasopressin and PMA (Figs. 4 and 5). We initially examined the formation of both intracellular and total (extracellular plus intracellular choline) (Fig. 4) in response to vasopressin. There was a rapid rise in the formation of total choline which reached a peak between 2 and 3 min at approx. 200–300% of control

Table 2. Effect of vasopressin on the incorporation of [^{3}H]choline into intracellular [^{3}H]phosphocholine in A10 VSMCC

Unlabelled cells were incubated with [^{3}H]choline (approx. 100000 d.p.m. per well) in the absence or presence of 30 nM-vasopressin (VP) at different times as outlined below. All reactions were terminated after 45 min and the intracellular formation of water-soluble choline metabolites was determined as outlined in the Methods section. Each value represents the mean ± S.D. of triplicate determinations from a single representative experiment, where $n = 4$.

Addition		[^{3}H]Phosphocholine (d.p.m.)
0 min	5 min	
[^{3}H]Choline	–	963 ± 86
[^{3}H]Choline + VP (30 nM)	–	585 ± 98
[^{3}H]Choline + VP	V_{1a} antagonist (10 μM)	572 ± 25
–	[^{3}H]Choline	755 ± 109
VP (30 nM)	[^{3}H]Choline	769 ± 61
VP (30 nM)	[^{3}H]Choline + V_{1a} antagonist	722 ± 80

values (Fig. 4*a*). After this time, both basal and stimulated values usually increased in parallel for the remainder of the time course. However, when intracellular choline formation was examined, we found that the kinetics of the signal were different (Fig. 4*b*). Although choline production increased rapidly in response to vasopressin, reaching an initial peak after 30–60 s (control, 344 ± 23 d.p.m.; vasopressin, 1161 ± 141 d.p.m.; from a single typical experiment where $n = 3$), stimulated values then declined, returning to basal within 5–10 min. No change was observed in the levels of phosphocholine in these experiments (results not shown). Experiments were performed to confirm this apparent desensitization phenomenon. Addition of excess V_{1a}-receptor antagonist (10 μM) after 5 min was without effect on the kinetics of vasopressin-stimulated choline formation (either intracellular or total), arguing against delayed receptor-stimulated activation of choline kinase. Furthermore, no corresponding changes in phosphocholine formation were observed under these conditions, again indicative of no receptor-dependent flux through this pathway (results not shown). When [^{3}H]choline was added to the incubation media 5 min after vasopressin stimulation, cellular uptake of [^{3}H]choline and the formation of [^{3}H]phosphocholine were identical in stimulated and control cells (Table 2). However, vasopressin stimulation resulted in a decrease in [^{3}H]phosphocholine levels in cells where [^{3}H]choline was added immediately before vasopressin, suggesting that synthesis of PtdCho was also transiently activated (Table 2).

The transient nature of the vasopressin-stimulated choline generation differed markedly from that obtained in response to PMA (Fig. 5). The phorbol ester elicited a quantitatively similar 2–3-fold increase in intracellular choline formation, which reached a peak after 2 min (control, 676 ± 94 d.p.m.; PMA, 1233 ± 58 d.p.m.; from a single typical experiment where $n = 3$). However, although the onset of the response was less rapid than that to vasopressin, it was more sustained, with [^{3}H]choline being elevated above basal values for at least 45 min (Fig. 5*a*). This was reflected in the total [^{3}H]choline formation (Fig. 5*b*), which, unlike the vasopressin response, increased in a linear manner over time. In addition, although phosphocholine formation was not affected by vasopressin stimulation (results not shown), [^{3}H]phosphocholine levels were decreased significantly in response to PMA, to approx. 50% of basal by 45 min (control, 7636 ± 593 d.p.m.; PMA, 2964 ± 305 d.p.m.) (Fig. 5*c*).

Fig. 6 shows the time course of vasopressin- and PMA-

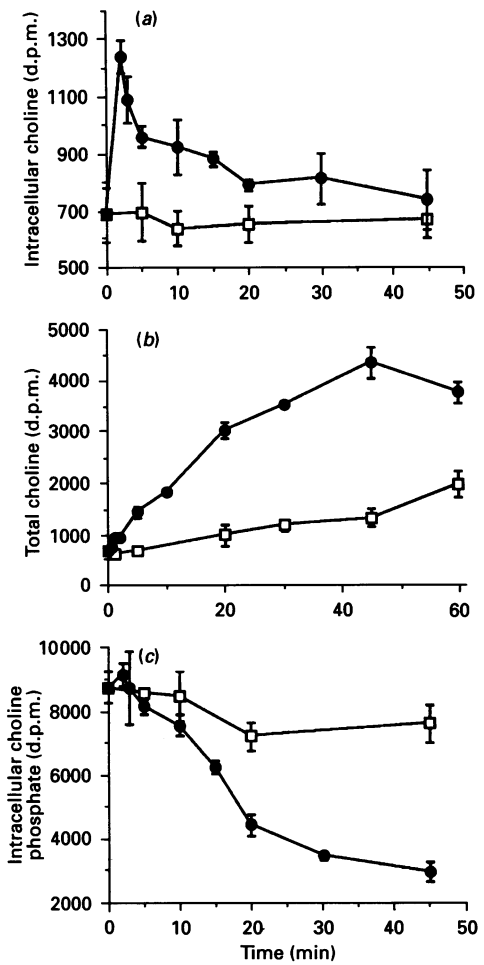

Fig. 5. Time course of PMA-stimulated intracellular (*a*) and total (*b*) choline and (*c*) intracellular phosphocholine formation in A10 VSMCC

Cells, prelabelled with [^{3}H]choline, were incubated with vehicle (□) or 100 nM-PMA (●) for the times indicated. Water-soluble choline metabolites were analysed as outlined in the Methods section. Each point represents the mean ± S.D. of triplicate determinations from a single typical experiment where $n = 3$.

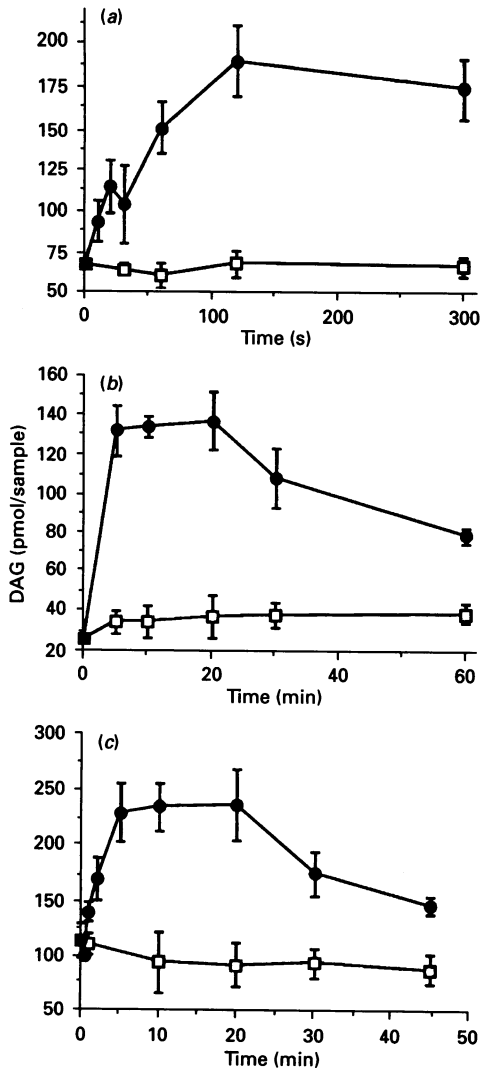

Fig. 6. Time course of vasopressin- and PMA-stimulated DAG formation in A10 VSMCC

Cells were incubated with vehicle (□) or stimulant (●) for the times indicated. In (*a*) and (*b*) the cells were stimulated with 100 nM-vasopressin, and in (*c*) with 100 nM-PMA. DAG levels were assayed as outlined in the Methods section. Each point represents the mean ± S.D. of triplicate determinations from a single typical experiment where $n = 4$.

stimulated DAG formation in A10 VSMCC. Vasopressin-stimulated DAG formation was biphasic, with an initial peak obtained after 30 s, at approx. 80% above basal values. Levels then either declined briefly or stayed constant, before increasing once more between 60 and 120 s (Fig. 6*a*). Maximum DAG levels were obtained between 2 and 5 min, routinely at 2.5–3-fold of basal values (control, 59 ± 8 pmol; vasopressin, 189 ± 21 pmol). DAG levels remained above control for up to 60 min, although they had decreased by about 50% over this time (see Fig. 6*b*). PMA also stimulated DAG formation in A10 VSMCC. However, there was no initial rapid phase of DAG formation, as stimulated by vasopressin, but rather a lag of some 30–60 s before the onset of the response (Fig. 6*c*). Maximum levels were usually reached between 5 and 10 min (Fig. 6*c*) and were sustained for up to 60 min (results not shown). The PMA-stimulated response was routinely approx. 40–60% of that obtained with vasopressin over a series of experiments.

The effect of preincubation with 30 mM-butanol on the kinetics of vasopressin- and PMA-stimulated DAG generation is shown in Fig. 7. In the presence of butanol, vasopressin-stimulated DAG formation was decreased by 40–50% between 1 and 10 min (Fig. 7*a*). However, as the stimulated formation of DAG decreased between 10 and 30 min, the effect of butanol was correspondingly diminished. This gave rise to a single sustained phase of DAG formation in the presence of butanol. As described above, PMA elicited a single phase of DAG formation which reached a peak between 2 and 5 min at approx. 180% of control values (see also Fig. 7*b*); after 20 min the signal declined, but remained above basal values for up to 45 min. However, preincubation with butanol decreased the response to PMA by 75–80% at all time points examined. Control experiments were performed to rule out non-specific effects of butanol on both inositol and choline phospholipid hydrolysis. Addition of 30 mM-butanol had no effect on vasopressin-stimulated [^{3}H]IP accumulation or [^{3}H]choline or [^{3}H]phosphocholine formation (results not shown).

The effect of preincubation with 100 μM of the PKC inhibitor Ro-31-8220 on vasopressin- and PMA-stimulated DAG formation was examined. This is the concentration required to inhibit fully vasopressin-stimulated PLD activity in A10 VSMCC (Plevin

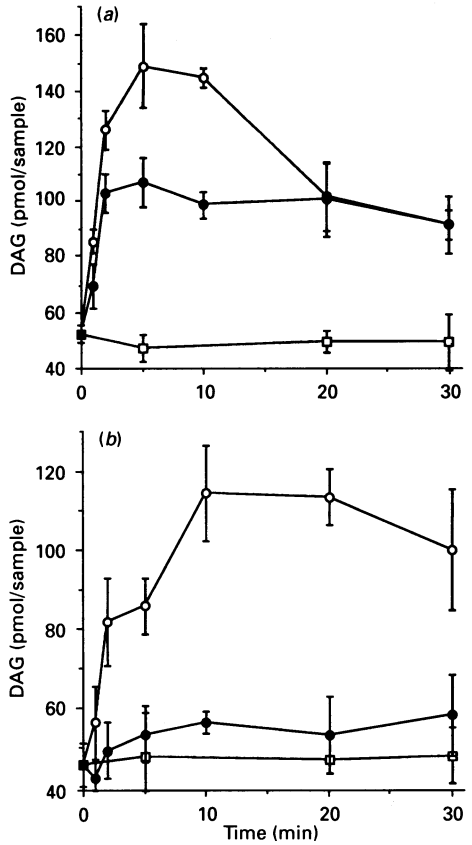

Fig. 7. Effect of butanol pretreatment on the time course of vasopressin- and PMA-stimulated DAG formation in A10 VSMCC

Cells were preincubated with buffer (○) or buffer containing 30 mM-butanol (●) for 5 min, then further incubated with 100 nM-vasopressin (*a*) or 100 nM-PMA (*b*). Each point represents the mean ± S.D. of triplicate determinations from a single experiment where $n = 4$. Basal values (□) shown here are in the absence of butanol; basal values in the presence of butanol were not significantly different and are omitted for the sake of clarity.

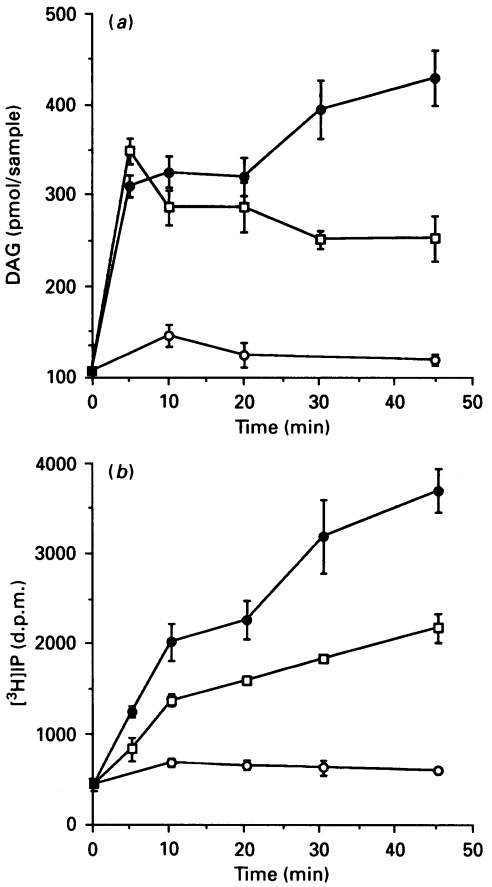

Fig. 8. Effect of Ro-31-8220 on prolonged vasopressin-stimulated DAG formation and [^{3}H]IP accumulation in A10 VSMCC

Cells were preincubated with buffer containing vehicle (0.1% dimethyl sulphoxide) (□) or 100 μM-Ro-31-8220 (●) for 5 min before addition of 100 nM-vasopressin for the times indicated; (○) represents control incubations. Each point represents the mean ± S.D. of triplicate determinations from a single experiment where $n = 3$–5.

et al., 1991*b*). In preliminary experiments, Ro-31-8220 inhibited the PMA-stimulated response by approx. 80–90% at 2 and 5 min (2 min: control, 46.9 ± 8.0 pmol/sample; PMA, 99.0 ± 5.3 pmol/sample; PMA + Ro-31-8220, 55.0 ± 5.3 pmol/sample; data from a single typical experiment where $n = 4$). However, the compound had much less effect on vasopressin-stimulated DAG formation, decreasing it by only 30–40% at 2 min (control, 46.9 ± 8.0 pmol/sample; vasopressin, 136.0 ± 8.5 pmol/sample; vasopressin + Ro-31-8220, 96.0 ± 15.0 pmol/sample; data from a single typical experiment where $n = 4$). The effect of Ro-31-8220 on prolonged vasopressin-stimulated DAG formation was also assessed (Fig. 8). Although the PKC inhibitor again decreased vasopressin-stimulated DAG formation by approx. 20% at 5 min, during more prolonged incubation times (30 and 45 min) the compound significantly potentiated DAG formation ($P < 0.05$ for 30 min and 45 min, for comparison of vasopressin stimulation in the presence or absence of Ro-31-8220; $n = 4$). Ro-31-8220 also enhanced vasopressin-stimulated [^{3}H]IP accumulation (Fig. 8*b*).

The agonist-dependency of sustained DAG formation is shown in Fig. 9. After addition of the V_{1a}-receptor antagonist after 5 min of agonist stimulation, the formation of DAG is significantly decreased. DAG levels fell to near basal values within 30–45 min (at 45 min: vasopressin, 152.0 ± 9.0 pmol/sample; vasopressin + antagonist, 66.5 ± 9.0 pmol/sample; control,

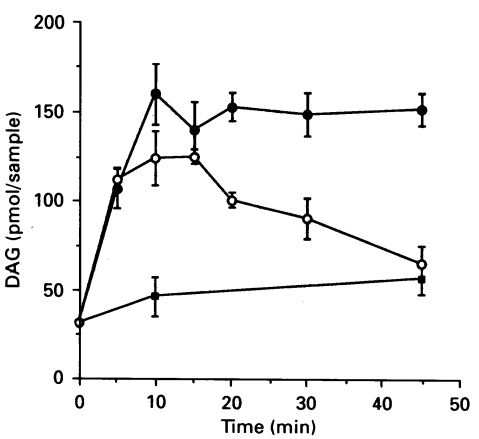

Fig. 9. Effect of V_{1a}-receptor antagonist on the time course of vasopressin-stimulated DAG formation in A10 VSMCC

Cells were incubated with vehicle (■) or 30 nM-vasopressin (●, ○) for the times indicated in the presence (○) or absence (●) of the V_{1a} antagonist (5 μM), added after 5 min initial stimulation. Each point represents the mean ± S.D. of triplicate determinations from a single representative experiment where $n = 4$.

66.3 ± 9.6 pmol/sample; data from a single typical experiment where $n = 4$). A similar phenomenon was observed if the antagonist was added after 10 min of agonist stimulation (results not shown).

DISCUSSION

In this study we sought to examine the relationship between the hydrolysis of PtdIns(4,5)P_2 and PtdCho and the formation of DAG in vasopressin-stimulated A10 VSMCC. Vasopressin has been shown to stimulate the hydrolysis of both PtdIns(4,5)P_2 and PtdCho through the activation of vasopressin V_{1a} receptors (Grillone *et al.*, 1988; Plevin *et al.*, 1991*b*). Preliminary experiments showed that *sn*-1,2-DAG mass formation in response to vasopressin was biphasic. This finding is consistent with a number of peptide receptor systems such as angiotensin II-stimulated VSMCC (Griendling *et al.*, 1986) and bombesin-stimulated Swiss 3T3 fibroblasts (Cook *et al.*, 1990). However, in those and other studies the second phase of DAG formation has been proposed to be derived from sustained PtdCho hydrolysis (Cook & Wakelam, 1989; Cook *et al.*, 1990; Lassegue *et al.*, 1991).

Initial experiments suggested that vasopressin-stimulated PtdIns-(4,5)P_2 hydrolysis was rapidly desensitized. Both vasopressin-stimulated increases in Ins(1,4,5)P_3 formation and the corresponding decrease in PtdIns(4,5)P_2 mass levels were transient. Re-challenge with vasopressin was without effect even under conditions where vasopressin had been competed off the receptor with a V_{1a}-receptor antagonist and the cells had then been re-challenged with excess peptide, suggesting that desensitization was not due to continual receptor occupancy. These findings are consistent with other receptor/G-protein/PLC systems (e.g. Palmer *et al.*, 1991) and indicate that desensitization of agonist-stimulated PtdIns(4,5)P_2 hydrolysis is a widespread phenomenon. However, although the kinetics of vasopressin-stimulated total [^{3}H]IP accumulation were biphasic, a finding again consistent with desensitization of PtdIns(4,5)P_2 hydrolysis (Cook *et al.*, 1990), the second phase was markedly affected by the addition of the V_{1a}-receptor antagonist (Fig. 2). This result could suggest that another inositol-containing phospholipid may be hydrolysed during prolonged receptor activation. A similar conclusion was made from studies in thyrotropin-releasing-hormone-stimulated pituitary cells (Imai & Gershengorn, 1986) and thrombin-stimulated platelets (Wilson *et al.*, 1985). In these studies PtdIns, but not PtdIns*P*, was shown to be hydrolysed in an agonist-dependent fashion. The alternative explanation would be that PtdIns(4,5)P_2 continues to be hydrolysed in vasopressin-stimulated A10 VSMCC, but at a lower rate, and that the activity of inositol lipid kinases is increased so that there is no apparent loss of PtdIns(4,5)P_2. This would also require the activity of Ins(1,4,5)P_3 phosphatase and/or kinase to be such that there would be no detectable accumulation of the second messenger (Fig. 1).

Our experiments also suggested that PLD activity was transient, being rapidly desensitized. This was determined initially by examining the rate of PtdBuOH accumulation stimulated by vasopressin. Furthermore, when A10 cells were preincubated with vasopressin before addition of butanol, we found that no further accumulation of PtdBuOH could be measured, indicating complete agonist desensitization of the agonist-stimulated response (Table 1). Since it was possible that the prevention of PtdBuOH formation by butanol did not allow the possible sustained formation of DAG and thus the associated reactivation of PKC, we analysed intracellular choline formation. However, this also demonstrated a transient signal in response to vasopressin, indicative of a desensitization phenomenon. However, intracellular choline formation was more sustained in response to PMA (Fig. 5), confirming that differences in the kinetics of intracellular choline formation reflect the desensitization properties of each stimulatory compound (MacNulty *et al.*, 1990; Plevin *et al.*, 1991*a*). The PLD desensitization phenomenon is not restricted to this agonist-receptor system; transient PLD activity has been recently shown in carbachol-stimulated 1321N1 cells (Martinson *et al.*, 1990) and in bombesin-stimulated Swiss 3T3 fibroblasts (R. Plevin & M. J. O. Wakelam, unpublished work). However, a more sustained PLD activity has recently been observed in platelet-derived-growth-factor-stimulated Swiss 3T3 cells (Plevin *et al.*, 1991*a*), suggesting that rapid desensitization of PLD-activated PtdCho hydrolysis may be a feature of receptors coupled via a G-protein to the effector.

We found that the formation of phosphocholine was not increased by vasopressin, a finding that agrees with a recent report by Welsh *et al.* (1990). This suggests that PLC does not catalyse PtdCho hydrolysis and that the activation of PLD is the major catalytic route of vasopressin-stimulated PtdCho hydrolysis in these cells. This is in contrast with previous studies in A10 VSMCC (Grillone *et al.*, 1988; Huang & Cabot, 1990), which have shown both vasopressin and PMA to stimulate modest increases in choline phosphate formation. In our study PMA stimulated a substantial decrease (60%) in [^{3}H]phosphocholine levels. This may be attributed to stimulated PtdCho synthesis, as previously shown for endothelin-1-stimulated Rat-1 fibroblasts (MacNulty *et al.*, 1990), again a result of sustained PLD activity. However, vasopressin did not stimulate such changes in choline and phosphocholine metabolism as observed for such sustained activators of PLD, i.e. PMA and endothelin-1. The consistent finding that PLD activity is transient clearly argues against PtdCho constituting the main source of sustained DAG in this tissue via this route.

Although vasopressin-stimulated PtdIns(4,5)P_2 and PtdCho hydrolysis was found to be transient, we sought to determine the contribution both pathways made to the totality of the DAG signal. Although the initial phase of agonist-stimulated DAG formation is derived from PtdIns(4,5)P_2, it is unlikely that this phospholipid contributes to the sustained generation of DAG, since PtdIns(4,5)P_2 hydrolysis was apparently transient and rapidly desensitized (Fig. 1). Furthermore, the DAG derived from this source appears to be rapidly metabolized by DAG kinase, as shown by the decrease in DAG levels when the agonist was removed from its receptor (Fig. 9). Since the formation of DAG from PLD-catalysed hydrolysis of PtdCho requires further conversion of the initial product, phosphatidic acid, by the action of phosphatidate phosphohydrolase, this pathway would be far more likely to be involved in the sustained phase. However, we found that only 40–50% of vasopressin-stimulated DAG formation between 1 and 10 min was derived from PLD-activated PtdCho hydrolysis, since DAG formation was only decreased by this amount in the presence of 30 mM-butanol. This conclusion was supported by several other observations. Firstly, PMA-stimulated DAG formation was approx. 50% of that stimulated by vasopressin. Secondly, this response was virtually abolished by either preincubation with butanol or pretreatment with PKC inhibitor, indicating that approximately this proportion of DAG is derived from PKC-mediated activation of PLD-catalysed PtdCho hydrolysis. Ro-31-8220 also inhibited initial vasopressin-stimulated DAG formation; however, the decrease was less than that observed for butanol, and at later times the inhibitor actually enhanced receptor-stimulated DAG formation (Fig. 8*a*). This effect was possibly due to an enhancement of vasopressin-stimulated PtdIns(4,5)P_2 hydrolysis, since Ro-31-8220 also enhanced vasopressin-stimulated [^{3}H]IP accumulation (Fig. 8*b*). Down-regulation or inhibition of PKC is well recognized to enhance vasopressin-stimulated inositol phosphate formation

(Brown *et al.*, 1990; Cook & Wakelam, 1991), owing to the inhibition of PKC-mediated negative feedback on receptor/G-protein coupling to PLC (Orellana *et al.*, 1987; Pfeilschifter & Bauer, 1987; Plevin *et al.*, 1990). Thus the magnitude and duration of DAG formation from PtdIns(4,5)P_2 hydrolysis would be enhanced by PKC inhibitors in a similar manner, as recently shown for thombin-stimulated platelets (Bishop *et al.*, 1990).

However, under normal conditions it is possible that sustained DAG is derived predominantly from a source other than PtdIns(4,5)P_2 and PtdCho hydrolysis. This source could be PtdIns; this lipid is implicated by the finding that both [^{3}H]IP accumulation and DAG formation are dependent on continual receptor activation. The potential for PtdIns or PtdIns*P* to provide the source of sustained DAG formation has been previously suggested for angiotensin II-stimulated VSMCC (Griendling *et al.*, 1986, 1987) and implicated in a number of other receptor systems (Imai & Gershengorn, 1986; Wilson *et al.*, 1985). However, the results in the present paper would also be consistent with a decreased rate of agonist-stimulated PtdIns(4,5)P_2 hydrolysis together with rapid PtdIns and PtdIns*P* phosphorylation and metabolism of Ins(1,4,5)P_3. The emphasis in the literature on agonist-stimulated PtdCho hydrolysis has led to the general assumption, questioned by the results in the present paper, that the majority of agonists utilize this particular pathway to sustain DAG formation. However, in addition to the results presented here, recent studies have suggested that a number of pathways may contribute to sustained formation of DAG in both an agonist- and tissue-specific manner. In carbachol-stimulated 1321N1 cells (Martinson *et al.*, 1990) and thrombin- or epidermal-growth-factor-stimulated fibroblasts (Pessin & Raben, 1989; Pessin *et al.*, 1990), DAG formation was reported to be wholly derived from PLD-activated PtdCho hydrolysis, whereas in bombesin-stimulated fibroblasts PtdCho accounted for only 30% of the DAG formed after 15 min (Cook *et al.*, 1991). Rossi *et al.* (1991) have also shown that in phagocytotic neutrophils sustained formation of DAG is derived from glucose via a synthetic pathway *de novo*, with little contribution from either PtdIns(4,5)P_2 or PtdCho hydrolysis.

The findings described in the present paper also have significant implications regarding the functional role of DAG in vascular smooth muscle cells. A recent report by Leach *et al.* (1991) has shown that only the DAG species derived from PtdIns(4,5)P_2 hydrolysis sustain the activation of the main isoforms of PKC (i.e. α, β and γ). However, it has also been suggested that species of DAG derived from PtdCho may activate the Ca^{2+}-independent isoforms of PKC (Thompson *et al.*, 1991). Thus the kinetics of phospholipid and PtdCho hydrolysis may be crucial in determining the patterns of PKC activation in response to a particular class of agonist. In VSMCC, PKC is considered to have multiple roles in intracellular events, including the regulation of sustained contraction (Haller *et al.*, 1990). Thus the continual hydrolysis of inositol-containing phospholipids may be a mechanism by which prolonged activation of the main isoforms of PKC is achieved in smooth-muscle cells.

We thank Andrew Paterson for development of and advice on the use of the DAG mass assay. This work was supported by grants from the Cancer Research Campaign and the Wellcome Trust.

Received 17 January 1992; accepted 7 February 1992

REFERENCES

Billah, M. M. & Anthes, J. C. (1990) Biochem. J. **269**, 281–291
Bishop, R. W., Ganong, B. R. & Bell, R. M. (1986) J. Biol. Chem. **261**, 6993–7000
Bishop, R. W., August, J., Petrin, J. M. & Pai, J.-K. (1990) Biochem. J. **269**, 465–473
Brown, K. D., Littlewood, C. J. & Blakely, D. M. (1990) Biochem. J. **270**, 557–560
Cook, S. J. & Wakelam, M. J. O. (1989) Biochem. J. **263**, 581–587
Cook, S. J. & Wakelam, M. J. O. (1991) Biochim. Biophys. Acta **1092**, 265–272
Cook, S. J., Palmer, S., Plevin, R. & Wakelam, M. J. O. (1990) Biochem. J. **265**, 617–620
Cook, S. J., Briscoe, C. P. & Wakelam, M. J. O. (1991) Biochem. J. **280**, 431–438
Ford, D. A. & Gross, R. W. (1990) J. Biol. Chem. **265**, 12880–12886
Griendling, K. K., Rittenhouse, S. E., Brock, T. A., Ekstein, L. S., Gimbrone, M. A., Jr. & Alexander, R. W. (1986) J. Biol. Chem. **261**, 5901–5906
Griendling, K. K., Delafontaine, P., Rittenhouse, S. E., Gimbrone, M. A., Jr. & Alexander, R. W. (1987) J. Biol. Chem. **262**, 14555–14562
Grillone, L. R., Clark, M. A., Godfrey, R. W., Stassen, F. & Crooke, S. T. (1988) J. Biol. Chem. **263**, 2658–2663
Haller, H., Smallwood, J. I. & Rasmussen, H. (1990) Biochem. J. **270**, 375–381
Huang, C. & Cabot, M. C. (1990) J. Biol. Chem. **265**, 14858–14863
Imai, A. & Gershengorn, M. C. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 8540–8544
Lassegue, B., Alexander, R. W., Clark, M. & Greindling, K. K. (1991) Biochem. J. **276**, 19–25
Leach, K. L., Ruff, V. A., Wright, T. M., Pessin, M. S. & Raben, D. M. (1991) J. Biol. Chem. **266**, 3215–3221
MacNulty, E. E., Plevin, R. & Wakelam, M. J. O. (1990) Biochem. J. **272**, 761–766
Martinson, E. A., Trivilas, I. & Brown, J. H. (1990) J. Biol. Chem. **265**, 22282–22287
Nishizuka, Y. (1984) Nature (London) **308**, 693–697
Ohanian, J., Ollershaw, J., Collins, P. & Heagerty, A. (1990) J. Biol. Chem. **265**, 8921–8928
Orellana, S., Solski, P. A. & Brown, J. H. (1987) J. Biol. Chem. **262**, 1638–1643
Palmer, S., Hughes, K. T., Lee, D. Y. & Wakelam, M. J. O. (1989) Cell Signalling **1**, 147–156
Palmer, S., Plevin, R. & Wakelam, M. J. O. (1991) Biochem. Soc. Trans. **19**, 1005
Paterson, A., Plevin, R. & Wakelam, M. J. O. (1991) Biochem. J. **280**, 829–830
Pessin, M. S. & Raben, D. M. (1989) J. Biol. Chem. **264**, 8729–8738
Pessin, M. S., Baldassare, J. J. & Raben, D. M. (1990) J. Biol. Chem. **265**, 7959–7966
Pfeilschifter, J. & Bauer, C. (1987) Biochem. J. **248**, 209–215
Plevin, R., Palmer, S., Gardner, S. D. & Wakelam, M. J. O. (1990) Biochem. J. **268**, 605–610
Plevin, R., Cook, S. J., Palmer, S. & Wakelam, M. J. O. (1991*a*) Biochem. J. **279**, 559–565
Plevin, R., Stewart, A., Paul, A. & Wakelam, M. J. O. (1991*b*). Br. J. Pharmacol. **104**, 39p
Rossi, F., Grzeskowiak, M., Bianca, V. D. & Sbarbati, A. (1991) J. Biol. Chem. **266**, 8034–8038
Sunako, M., Kawahara, Y., Hirata, K.-I., Tsuda, T., Yokoyama, M., Fukuzaki, H. & Taki, Y. (1990) Hypertension **15**, 84–88
Thompson, N. T., Bonser, R. W. & Garland, L. G. (1991) Trends Pharmacol. Sci. **12**, 404–408
Welsh, C. J., Schmeichel, K., Cao, H. & Chabbott, H. (1990) Lipids **25**, 675–684
Wilson, D. B., Neufeld, E. J. & Majerus, P. W. (1985) J. Biol. Chem. **260**, 1046–1051