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Vasopressin-stimulated [^{3}H]-inositol phosphate and [^{3}H]-phosphatidylbutanol accumulation in A10 vascular smooth muscle cells

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1 The characteristics of vasopressin-stimulated phosphatidylinositol 4,5 bisphosphate (PtdIns(4,5)P_2) and phosphatidylcholine (PtdCh) hydrolysis were examined in A10 vascular smooth muscle cells (VSMC), by assessing the formation of [^{3}H]-inositol phosphates ([^{3}H]-IP) and the accumulation of the phospholipase D (PLD) specific product, [^{3}H]-phosphatidylbutanol ([^{3}H]-PtdBuOH).

2 Vasopressin ([Arg^8]-VP) and a number of related analogues stimulated the accumulation of [^{3}H]-IP and [^{3}H]-PtdBuOH with similar EC_{50} values, generating the same rank order of potency for each response (Arg^8-VP = vasotocin = Lys^8-VP $\gg$ oxytocin).

3 Inhibition of vasopressin-stimulated [^{3}H]-IP and [^{3}H]-PtdBuOH accumulation by the V_{1a} receptor antagonists, Des-Gly^9[β-mercapto-β,β,-cyclopentamethylene propionyl, O-Et-Tyr^2,Val^4,Arg^8]-vasopressin generated similar IC_{50} values suggesting that both these responses are mediated through the activation of a single V_{1a} receptor subtype.

4 The onset of vasopressin-stimulated inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3) mass formation preceeded [^{3}H]-PtdBuOH accumulation indicating that PtdCh hydrolysis was activated subsequent to PtdIns(4,5)P_2 breakdown.

5 The protein kinase C (PKC) activator, tetradecanoylphorbol acetate (TPA) also stimulated [^{3}H]-PtdBuOH accumulation. Preincubation with the PKC inhibitor Ro-31-8220 abolished both TPA- and vasopressin-stimulated [^{3}H]-PtdBuOH, suggesting that the intermediate activation of protein kinase C is involved in the regulation of PLD by vasopressin.

6 Pretreatment of the A10 VSMC with Ro-31-8220 (100 μM) also potentiated vasopressin-stimulated Ins(1,4,5)P_3 mass formation. Therefore stimulation of PKC may have opposing roles in the regulation of agonist activation of PLC and PLD.

7 Preincubation of the cells with EGTA, verapamil, or the receptor-operated calcium channel antagonist, SK&F 96365, reduced vasopressin-stimulated [^{3}H]-PtdBuOH accumulation by approximately 30%, suggesting that influx of calcium has a significant role to play in the regulation of vasopressin-stimulated PLD activity.

Keywords: Vasopressin; phosphatidylinositol hydrolysis; phosphatidylcholine hydrolysis; protein kinase C; phospholipase D

Introduction

A number of vasoconstrictors stimulate the phospholipase C (PLC) catalysed hydrolysis of phosphatidylinositol 4,5 bisphosphate (PtdIns(4,5)P_2) to generate the two second messengers inositol -1,4,5-trisphosphate (Ins(1,4,5)P_3) and sn-1,2-diacylglycerol (DAG). These molecules are thought to be involved in different stages of the contractile process. Ins(1,4,5)P_3 stimulates the initial release of calcium from internal stores, whilst DAG activates protein kinase C (PKC) (Berridge & Irvine, 1989; Nishizuka, 1984). PKC is thought to play a role in the maintenance of contraction, through the phosphorylation of proteins such as caldesmon and myosin light chain when $[Ca^{2+}]_i$ has returned to near basal levels (Rasmussen *et al.*, 1987).

In smooth muscles cells, it has been established that, for a number of agonists such as endothelin-1, angiotensin II and vasopressin, PtdIns(4,5)P_2 hydrolysis is rapidly desensitized and $[Ca^{2+}]_i$ levels fall, whereas DAG levels remain elevated (Greindling *et al.*, 1986; Sunako *et al.*, 1990). Sustained DAG is thought to be derived from the hydrolysis of another phospholipid phosphatidylcholine (PtdCh) (see review by Billah & Anthes, 1990). Phosphatidylcholine hydrolysis has been demonstrated in a number of systems including vasopressin-stimulated hepatocytes (Augert *et al.*, 1989), gonadotrophin releasing hormone-stimulated ovarian granulosa cells (Liscovitch & Amsterdam, 1989), bombesin- and vasopressin-(Cook & Wakelam, 1991) and platelet derived growth factor-stimulated (Plevin *et al.*, 1991) Swiss 3T3 fibroblasts. However, in vascular smooth muscle cells little is known about the manner in which this process is regulated, in particular with regards the potential roles of PKC and extracellular calcium, both of which have been suggested to regulate PtdCh hydrolysis in other tissues (Billah *et al.*, 1989; MacNulty *et al.*, 1990). It has also been proposed that in some tissues agonist-stimulated PtdCh hydrolysis may be regulated by a specific G-protein or possibly through activation of different receptor subtypes (Boccking *et al.*, 1987). We therefore sought to examine the possible pathways by which vasopressin-stimulated PtdCh hydrolysis in vascular smooth muscle cells.

A preliminary account of some of these findings has been presented to the British Pharmacological Society (Plevin *et al.*, 1991).

Methods

A10 rat vascular smooth muscle cells (VSMC) were maintained in Dulbecco's modified Eagle's medium (DMEM) con-

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taining 15% foetal calf serum (FCS) at 37°C in a humidified atmosphere of air/CO_2 (19:1). For experiments cells were seeded in 24-well plates and labelled for 72 h with either [^{3}H]-inositol (Sp.Act. 20.0 kBq mmol^{-1}) in inositol free-DMEM containing 3% dialysed FCS, in DMEM containing 3% FCS with [^{3}H]-palmitic acid (20.7 kBq mmol^{-1}) or [^{3}H-methyl]-choline chloride (17.8 kBq mmol^{-1}). On the day of the experiments the labelling medium was removed and the cells were washed twice with 0.5 ml of Hank's buffered saline, pH 7.4, containing 1% (w/v) bovine serum albumin (fraction V) and 10 mM-glucose (HBG), and incubated for 30 min in this buffer. For inositol phosphate experiments the cells were incubated for 10 min with HBG containing 10 mM-LiCl (HBG/LiCl) and the stimulation was then carried out in a final volume of 0.25 ml for 30 min at 37°C. The reaction was terminated by the addition of 50 µl 10% (v/v) $HClO_4$. For the measurement of intracellular choline formation, the cells were incubated with agonist for the times indicated, the media aspirated and the reaction terminated by the addition of 0.5 ml ice cold methanol. For the assay of PLD-stimulated PtdBuOH accumulation, cells prelabelled with [^{3}H]-palmitic acid were preincubated with 30 mM butanol for 5 min before initiation of the reaction. The reaction was terminated by rapid aspiration followed by the addition of 0.5 ml of ice cold methanol.

For analysis of total labelled inositol phosphates, the cell extracts were scraped, washed with a further 100 µl of 10% (v/v) $HClO_4$ and then transferred to vials. The samples were neutralised with 1.5 M KOH/60 mM HEPES and the water-soluble inositol phosphates assayed by batch chromatography on Dowex-1 formate columns as previously described (Plevin *et al.*, 1990). For measurement of choline metabolites the cells were scraped, washed (0.25 ml methanol) and the extracts transferred to plastic vials. Chloroform (0.3 ml) was added and the samples allowed to extract at room temperature for 30 min. The phases were split by the addition of chloroform (0.5 ml) and water (0.5 ml) followed by centrifugation. An aliquot of the upper aqueous phase was analysed for glycerophosphocholine, choline phosphate and choline by cation exchange chromatography upon Dowex-50W-H^+ columns (Cook & Wakelam, 1989). For measurement of PtdBuOH formation the cell extracts were treated as above except glass vials were used throughout. The aqueous phase was removed and the lower phase dried down under vacuum. The sample was dissolved in chloroform/methanol 19:1 (v/v) and applied to Whatman LK5DF plates as described by Randall *et al.* (1990). The resolving solvent consisted of the upper phase of 2,2,4,-trimethylpentane/ethyl acetate/acetic acid/H_2O (50/110/20/100, v/v). The location of the peak was established by co-migration with a [^{3}H]-Ptd-butanol standard (R_F value 0.4). Preliminary experiments confirmed the dose-dependency of [^{3}H]-PtdBuOH formation upon butan-1-ol concentration (results not shown).

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

Dose-response curves were fitted to a logistic equation by an iterative fitting procedure (Delean *et al.*, 1980). Statistical analysis was performed using Student's *t* test. Results are given ± s.e.mean.

All radiolabelled compounds were obtained from Amersham International (Amersham, Bucks). All other compounds were of the highest grades commercially available. Ro-31-8220 and SK&F 96365 (1-{β-[3-(4-methoxyphenyl) propoxy]-4-methoxyphenethyl}-1H-imidazole hydrochloride) were gifts from Roche Products Ltd. and Dr J.E. Merritt, Smith Kline & Beecham Ltd respectively.

Results

Initial experiments showed that vasopressin-stimulated PtdCh hydrolysis in A10 smooth muscle cells, as measured by the formation of water soluble choline in cells prelabelled with [^{3}H]-choline. An approximate 2-3 fold increase in intracellular [^{3}H]-choline formation was obtained upon stimulation with 100 nM of the peptide ((d.p.m. ± s.e.mean) control = 385 ± 21; vasopressin = 1221 ± 57, $n = 5$). In contrast, there was no significant increase in choline phosphate formation (control = 10889 ± 1594 d.p.m.; vasopressin = 9549 ± 534 d.p.m., $n = 5$) suggesting that vasopressin stimulated PtdCh hydrolysis by a PLD-catalysed mechansim. This was confirmed by measuring vasopressin-stimulated formation of phosphatidylbutanol in cells preincubated with 30 mM butanol. A 6–8 fold increase in [^{3}H]-PtdBuOH accumulation was obtained over a 5 min incubation period (control = 1130 ± 73 d.p.m.; vasopressin = 8248 ± 999 d.p.m., $n = 5$). Vasopressin also stimulated the accumulation of [^{3}H]-IP in A10 VSMCC. Over a series of experiments vasopressin stimulated a 4–6 fold increase in [^{3}H]-IP accumulation over a 30 min period (control = 796 ± 141 d.p.m.; vasopressin = 3129 ± 543 d.p.m., $n = 6$). In order to generate sufficient radioactivity associated with [^{3}H]-IP and [^{3}H]-PtdBuOH respectively, times of 30 and 5 min were selected for pharmacological studies. The potential cellular toxicity of butanol also necessitated measuring [^{3}H]-PtdBuOH at relatively short time points. Ins(1,4,5)P_3 mass levels were measured in the absence of LiCl since in a number of high performance liquid chromatography (h.p.l.c.) studies inclusion of lithium resulted in a biphasic accumulation of [^{3}H]-Ins(1,4,5)P_3 (results not shown). Butanol was without effect upon the onset of vasopressin-stimulated Ins-(1,4,5)P_3 accumulation and was thus omitted from further experiments (results not shown).

The pharmacological characteristics of vasopressin ([Arg^8]-vasopressin)-stimulated PLC and PLD activity were examined. Vasopressin stimulated both [^{3}H]-IP and [^{3}H]-PtdBuOH formation in the low nanomolar range and the EC_{50} values for [^{3}H]-IP and [^{3}H]-PtdBuOH accumulation were similar for a number of vasopressin analogues (see Table 1). A rank order of potency of [Arg^8]-vasopressin = vasotocin = [Lys^8]-vasopressin >> oxytocin was obtained in each case. In addition to being less potent than other analogues, oxytocin was found to be a partial agonist, only eliciting 60–70% of the maximum vasopressin response. The receptor specificity was further examined by assessing the effect of the V_{1a} antagonist on vasopressin-stimulated [^{3}H]-IP and [^{3}H]-PtdBuOH accumulation (Figure 1). Similar IC_{50} values were obtained for the inhibition of both responses ([^{3}H]-IP = 37.2 ± 12 nM; [^{3}H]-PtdBuOH = 20.6 ± 9 nM, $n = 3$). These results indicated that both PLC and PLD are controlled through activation of a single V_{1a} subtype.

The kinetics of vasopressin-stimulated Ins(1,4,5)P_3 mass [^{3}H]-IP accumulation and [^{3}H]-PtdBuOH accumulation is shown in Figures 2 and 3. Ins(1,4,5)P_3 formation increased rapidly in response to vasopressin, reaching a peak between 10–20 s at 6–7 fold greater than basal (Figure 2). Stimulated levels then fell rapidly between 30–120 s, returning to basal values within 5 min. By comparison, vasopressin-stimulated [^{3}H]-PtdBuOH formation was only detectable after a lag of some 10–15 s. Accumulation then increased in a linear manner before reaching a maximum between 2–3 min. Stimulated PtdBuOH levels remained constant for a further 60 min suggesting that PLD activity was transient (Figure 3a). This contrasted with the time course of [^{3}H]-IP accumulation (Figure 3b). Following a lag time of approximately 2 min, inositol phosphate accumulation increased in a linear manner for between 45–60 min after which no further accumulation was observed (Figure 3b).

The PKC activating phorbol ester, tetradecanoylphorbol acetate (TPA), also stimulated PLD activity in a dose-dependent manner (Figure 4) with an EC_{50} value of 10.96 ± 3.16 nM ($n = 3$). This value is in close agreement with that

Table 1 EC_{50} values for stimulated [^{3}H]-inositol phosphate ([^{3}H]-InsP) and [^{3}H]-phosphatidylbutanol ([^{3}H]-PtdBuOH) formation in A10 VSMC in response to vasopressin analogues

Analogue	*EC_{50} (nM)*	
	[^{3}H]-InsP	*[^{3}H]-PtdBuOH*
[Arg^8]-vasopressin	3.37 ± 0.53	1.84 ± 0.64
Vasotocin	2.49 ± 0.21	2.78 ± 1.3
[Lys^8]-vasopressin	3.47 ± 0.43	2.78 ± 1.5
Oxytocin	626.00 ± 17.0	668.00 ± 18.0

Cells prelabelled as outlined in the methods section were incubated with increasing concentrations of analogue for 30 min or 5 min, then assayed for [^{3}H]-InsP or [^{3}H]-PtdBuOH accumulation respectively. Each value represents the mean ± s.e.mean obtained from at least 3 separate experiments performed in triplicate ($n = 3-5$). Basal values for [^{3}H]-IP accumulation were between 700–1250 d.p.m. and stimulation were routinely 4–600% of basal values. Basal values for [^{3}H]-PtdBuOH accumulation were between 1200 and 1860 d.p.m. Vasopressin stimulations were 5–700% of basal value

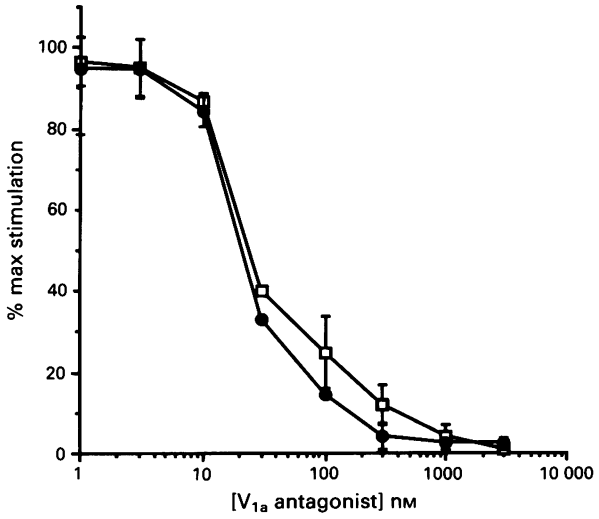

Figure 1 The effect of the V_{1a} receptor antagonist on vasopressin-stimulated [^{3}H]-inosoitol phosphate ([^{3}H]-IP) and [^{3}H]-phosphatidylbutanol ([^{3}H]-PtdBuOH) accumulation in A10 cells. Cells prelabelled as in the method section were incubated with 10 nM vasopressin in the presence of increasing concentrations of Des-Gly^9-[β-mercapto-β,β-cyclopentamethylene-propionyl^1, O-Et-Tyr^2, Val^4, Arg^8]-vasopressin as described in the legend to Table 1, and then assayed for [^{3}H]-IP (●) and [^{3}H]-PtdBuOH (□). Each point is the mean (s.e.mean shown by vertical bars) where $n = 3$. Basal values varied between 1000–1300 d.p.m. for [^{3}H]-PtdBuOH accumulation and 650–900 d.p.m. for [^{3}H]-IP accumulation. Vasopressin stimulated a 3–5 fold increase in [^{3}H]-IP accumulation and a 5–7 fold increase in [^{3}H]-PtBuOH accumulation.

obtained for TPA activation of PKC in a number of tissues (Nishizuka, 1984). The effect of preincubation with the PKC inhibitor Ro-31-8220 (Davis *et al.*, 1989), upon both TPA- and vasopressin-stimulated PLD activity is shown in Figure 5. TPA-stimulated PtdBuOH accumulation was completely abolished by low μM concentrations of Ro-31-8220, with an IC_{50} value of 1.85 ± 0.79 μM ($n = 3$). Pretreatment with Ro-31-8220 also abolished vasopressin-stimulated [^{3}H]-PtdBuOH accumulation. However, agonist stimulation was approximately 15 fold less sensitive to Ro-31-8220 pretreatment, with full inhibition being obtained at approximately 100 μM ($IC_{50} = 27.5 \pm 0.65$ μM, $n = 3$). The effect of the PKC inhibitor on vasopressin-stimulated Ins(1,4,5)P_3 formation was also examined (Figure 6). Preincubation with 100 μM Ro-31-8220 significantly enhanced maximal vasopressin-stimulated Ins(1,4,5)P_3 formation by approximately 50%, but was without effect upon the basal level (control = 2.16 ± 0.49 – 9.1 ± 1.3 pmol; Ro-31-8220 = 2.06 ± 0.46 – 15.5 ± 1.0 pmol at 20 s). In addition, stimulated values returned to basal in the presence of Ro-31-8220 more slowly than in control cells. A two fold increase in Ins(1,4,5)P_3 levels was still observed after 5 min following preincubation with this compound (1.85 ± 0.28 vs 3.6 ± 0.3 pmol).

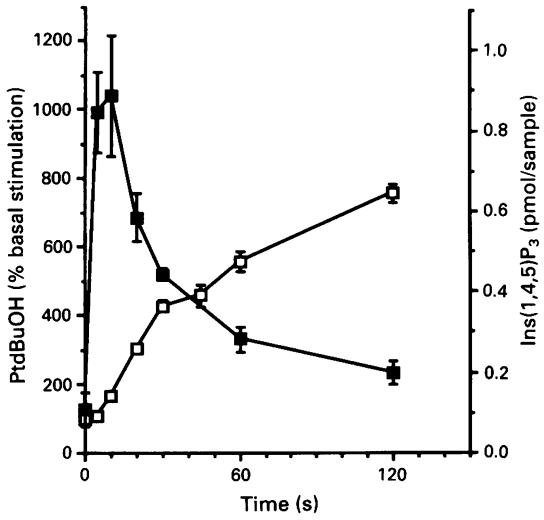

Figure 2 The onset of vasopressin-stimulated inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3) formation and [^{3}H]-phosphatidylbutanol ([^{3}H]-PtdBuOH) accumulation in A10 cells. Cells were incubated with vasopressin (100 nM) for the times indicated then Ins(1,4,5)P_3 mass (■) measured; [^{3}H]-palmitate labelled cells were stimulated at the times indicated and the formation of [^{3}H]-PtdBuOH determined (□). Each point represents the mean (s.e.mean shown by vertical bars) where $n = 4$ (12 observations). Basal values for [^{3}H]-PtdBuOH accumulation were 950–1500 d.p.m. over 4 experiments.

The effect of removing extracellular calcium on vasopressin-stimulated PLD activity is shown in Table 2. Reducing the external calcium concentration to 100 nM by buffering with EGTA resulted in an approximate 30% decrease in vasopressin-stimulated accumulation of [^{3}H]-PtdBuOH (Table 2). TPA-stimulated [^{3}H]-PtdBuOH formation was unaffected by this treatment. The calcium ionophore A23187 also stimulated the accumulation of [^{3}H]-PtdBuOH in control cells. However, this was only approximately 30% of the vasopressin response and virtually abolished by calcium chelation with EGTA. The protein kinase C inhibitor, Ro-31-8220, abolished the A23187 response, suggesting that calcium regulation of PLD activity is mediated through an action upon PKC. Ro-31-8220 (30 μM) also reduced vasopressin-stimulated PtdBuOH accumulation by approximately similar amounts (50 and 66% respectively). In a number of additional experiments the PKC inhibitor was also found to be equally effectively against vasopressin-stimulated PLD activity in both control and in calcium-free conditions (IC_{50} values (μM) control = 28.51 ± 7.2; 100 mM Ca^{2+} = 31.1 ± 6.3, $n = 3$) suggesting that the agonist activation of PKC is not significantly compromised by the removal of extracellular calcium. A number of calcium channel antagonists were also without effect upon vasopressin-stimulated PLD activity (Table 2). This included the voltage-dependent calcium channel inhibitors, nifedipine and verapamil, and the putative receptor operated calcium antagonist, SK&F 96365 (Merrit *et al.*, 1990). Only at high micromolar concentrations of the drug was any significant inhibition observed and at these concentrations the drug was equally effective against K^+-

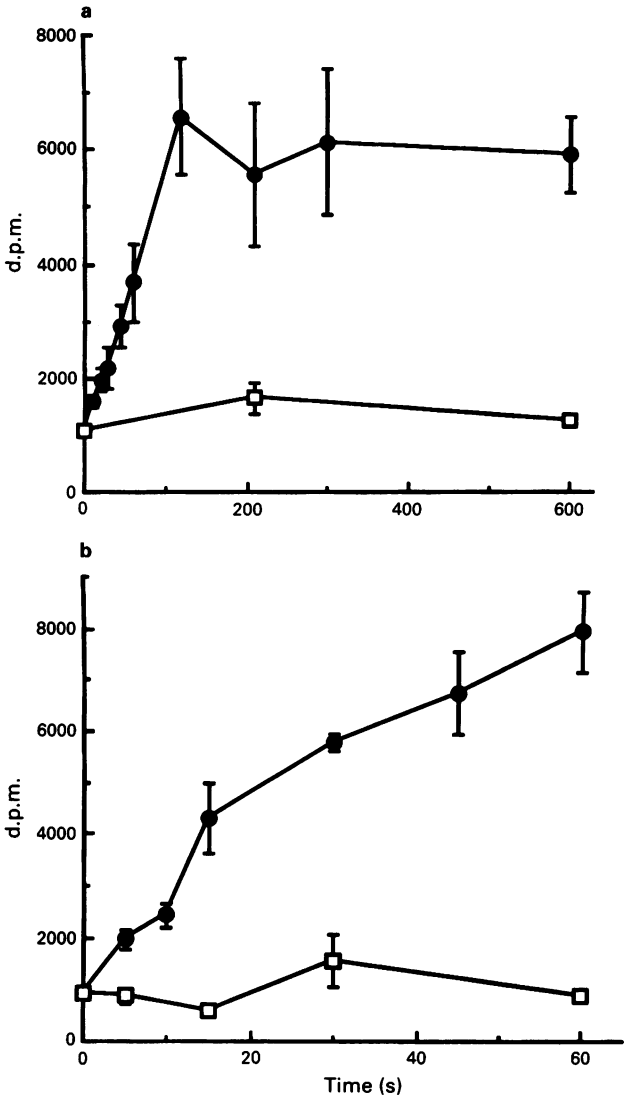

Figure 3 The kinetics of vasopressin stimulated [^{3}H]-phosphatidylbutanol ([^{3}H]-PtdBuOH) and [^{3}H]-inositol phosphate ([^{3}H]-IP) accumulation in A10 smooth muscle cells. Cells prelabelled as outlined in the Methods section were incubated with vehicle (□) or 100 nM vasopressin (●) for the time indicated then assayed for PtdBuOH (a) or [^{3}H]-IP accumulation (b). Each point represents the mean (s.d. shown by vertical bars) of triplicate determinations from a single representative experiment were $n = 3$.

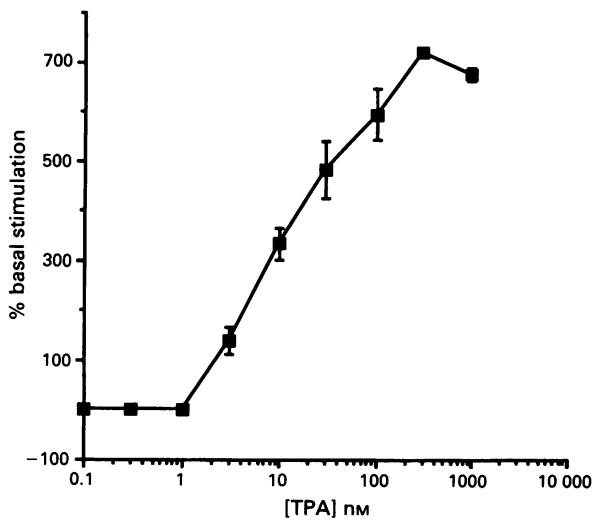

Figure 4 Tetradecanoylphorbol acetate (TPA) dose-dependent stimulation of [^{3}H]-phosphatidylbutanol ([^{3}H]-PtdBuOH) accumulation in A10 smooth muscle cells. Cells preincubated as outlined in the methods section were incubated with increasing concentrations of TPA for 15 min, then assayed for [^{3}H]-PtdBuOH formation. Each point represents the mean (s.e.mean shown by vertical bars) where $n = 3$. Basal values varied from 1100–1650 d.p.m.

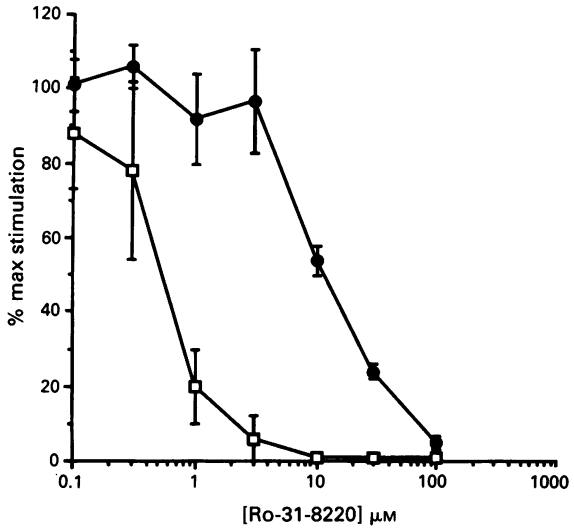

Figure 5 The effect of Ro-31-8220 on tetradecanoylphorbol acetate (TPA) and vasopressin-stimulated [^{3}H]-phosphatydlbutanol ([^{3}H]-PtdBuOH) accumulation in A10 cells. Cells prelabelled as outlined in the methods section were preincubated with increasing concentrations of Ro-31-8220 for 5 min, stimulated with 100 nM TPA (□) or vasopressin (●) for 15 min and then [^{3}H]-PtdBuOH formation determined. Each point represents the mean (s.e.mean shown by vertical bars) where $n = 4$. Basal values varied between 850–1600 d.p.m. and stimulated values ranged between 5–7 fold for vasopressin and 9–14 fold for TPA.

stimulated PLD activity. The time course of vasopressin-stimulated Ins(1,4,5)P_3 formation in control and reduced [Ca^{2+}] conditions is shown in Table 3. Preincubation with EGTA had no effect upon either the magnitude or duration of vasopressin-stimulated Ins(1,4,5)P_3 formation in A10 cells.

Discussion

In this study we examined the mechanisms by which PLD-catalysed PtdCh hydrolysis is regulated by vasopressin in the A10 smooth muscle cell line. Although this cell line has limited application as a model for smooth muscle contraction due to the loss of actin and myosin, the early events associated with this process such as calcium transients appear to be intact. Few studies have examined the mechanisms of agonist-stimulated PtdCh hydrolysis in smooth muscle, although both primary and secondary products of this pathway have been implicated in the initiation and maintenance of both smooth muscle contraction and proliferation (Ohanian *et al.*, 1990). Initial experiments measured the accumulation of the PLD-specific product phosphatidylbutanol. Although this assay is used routinely to measure agonist-stimulated PtdCh hydrolysis it is possible that PLD may catalyse the hydrolysis of other phospholipids such as phosphatidylserine and phosphatidylethanolamine to give the same product. However, in a number of fibroblast cell lines we have shown that [^{3}H]-palmitate is incorporated into these

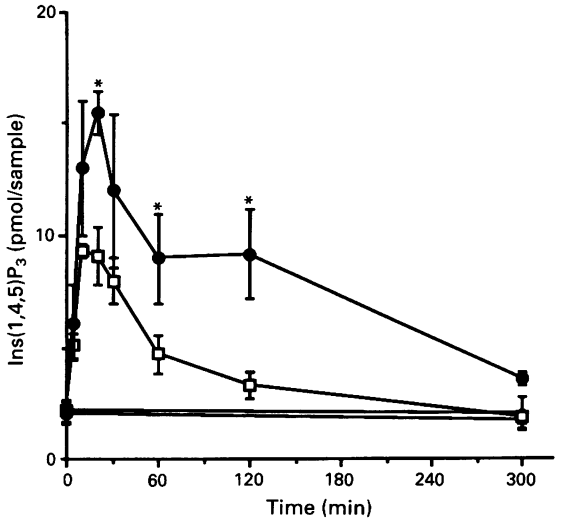

Figure 6 The effect of Ro-31-8220 on vasopressin-stimulated inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3) mass formation in A10 cells. Unlabelled cells were preincubated with vehicle (□) or 100 μM Ro-31-8220 (●) for 10 min then incubated with vasopressin (100 nM) for the times indicated and Ins(1,4,5)P_3 mass measured as described in the methods. Each point represents the mean (s.e.mean shown by vertical bars) where $n = 3$. *$P < 0.005$ vs control stimulation with vasopressin.

Table 2 The effect of extracellular calcium concentration reduction and calcium channel inhibition on [^{3}H]-phosphatidylbutanol ([^{3}H]-PtdBuOH) accumulation in A10 vascular smooth muscle cells.

	% basal stimulation	
	1.26 nM [Ca^{2+}]	*100 nM [Ca^{2+}]*
A. VP (100 nM)	540 ± 41	396 ± 23*
TPA	1033 ± 66	995 ± 50
A23187	238 ± 11	125 ± 8*
VP + Ro-31-8220 (30 μM)	248 ± 18	294 ± 17
A23187 + Ro-31-8220 (30 μM)	114 ± 4	ND
B. VP (100 nM)	568 ± 17	
VP + verapamil (10 μM)	548 ± 12	
+ nifedipine (5 μM)	571 ± 12	
+ SK&F 96365 (10 μM)	605 ± 26	
+ SK&F 96365 (100 μM)	358 ± 34*	
K^+ (80 mM)	338 ± 31	
+ SK&F 96365 (10 μM)	302 ± 22	
+ SK&F 96365 (100 μM)	184 ± 21*	

Prelabelled cells were preincubated with either HBG or HBG containing Ca^{2+} and 0.3 mM EGTA (free [Ca^{2+}] = 100 nM) or calcium channel antagonists for 15 min before addition of TPA (100 nM), vasopressin (VP) (100 nM), A23187 (5 μM) or 80 mM K^+ for 5 min. Each d.p.m. value represents the mean ± s.e.mean where $n = 4$ (12 observations). *$P < 0.05$ vs 1.26 mM [Ca^{2+}] stimulated with VP (**A**) or vs VP or K^+ stimulation (**B**). Basal values (d.p.m.), experiments (**A**) 1.26 mM [Ca^{2+}] = 787 ± 57; 100 nM [Ca^{2+}] = 874 ± 65; experiment (**B**) = 943 ± 88. For abbreviations, see text. ND = not done.

phospholipids to only a minor extent (Cook & Wakelam, unpublished results) and a similar finding has been obtained by Welsh *et al.* (1990) in A10 VSMC. Additional preliminary experiments also suggested that PtdCh was a major substrate for vasopressin-stimulated, PLD-catalysed hydrolysis, as measured by the formation of water soluble choline metabolites.

Table 3 The effect of extracellular calcium removal on vasopressin-stimulated inositol-1,4,5-trisphosphate (Ins(1,4,5) P_3) formation in A10 cells

	Ins(1,4,5)P_3 (pmol/sample)	
	1.26 mM [Ca^{2+}]	*100 nM [Ca^{2+}]*
Control (10 s)	2.40 ± 0.24	1.96 ± 0.45
Vasopressin (10 s)	11.60 ± 1.89	10.10 ± 2.0
Control (5 min)	1.96 ± 0.2	2.33 ± 0.2
Vasopressin (5 min)	3.20 ± 0.58	2.80 ± 0.75

Cells were preincubated in HBG or in HBG containing 0.3 mM EGTA (free [Ca^{2+}] = 100 nM) for 15 min then incubated with 100 nM vasopressin or vehicle for the times indicated. Ins(1,4,5)P_3 was measured as outlined in the methods. Each value represents the mean ± s.e.mean where $n = 3$.

We initially sought to characterize pharmacologically vasopressin-stimulated [^{3}H]-IP and [^{3}H]-PtdBuOH formation since it is believed that at least two vasopressin-V_1 receptor subtypes are expressed in mammalian tissues (Penit *et al.*, 1983; Jard *et al.*, 1986). In A10 and primary vascular smooth muscle cells a V_{1a} receptor subtype has been identified as mediating vasopressin-stimulated calcium influx and inositol phosphate formation (Doyle & Ruegg, 1985; Aiyar *et al.*, 1986). However, athough much attention has focused on the possibility that PLD and PLC may be regulated by distinct G-proteins, little attempt has been made to determine if PLC and PLD may be achieved by occupation of different receptor subtypes. This does not appear to be the case in A10 cells since the order of agonist potency was the same for both responses (Table 1) and the V_{1a} antagonist (Des-Gly^9-[β-mercapto-β,β-cyclopentamethylene-propionyl^1, O-Et-Tyr^2, Val^4, Arg^8]-vasopressin) yielded similar IC_{50} values in each case (Figure 1). There was also no indication of a high affinity receptor for oxytocin, indeed this peptide behaved as a weak, partial agonist, with about 60% of the efficacy of vasopressin (Table 1). Thus, both PLC and PLD appear to be stimulated by occupation of a V_{1a} receptor; however, an accurate receptor subtype definition can only be achieved by a more extensive comparison of agonist and antagonist potency orders (Jard *et al.*, 1986).

The kinetics of vasopressin stimulated Ins(1,4,5)P_3 accumulation were transient suggesting that the activation of PLC was not sustained. This is supported by a number of additional experiments which have shown a transient decrease in PtdIns(4,5)P_2 mass levels in response to vasopressin (Plevin & Wakelam, 1992). This finding argues strongly against the rapid removal of Ins(1,4,5)P_3 by 5-phosphatase and 3-kinase activity being the sole mechanism underlying the rapid return of Ins(1,4,5)P_3 accumulation to basal values. However, it is also possible that an agonist-stimulated flux through PtdIns and PtdIns(4)P may lead to a new steady state level of PtdIns(4,5)P_2. Therefore the possible sustained nature of PtdIns(4,5)P_2 hydrolysis may be impossible to delineate under the present assay conditions.

The apparent transient nature of PtdIns(4,5)P_2 hydrolysis argues against this lipid being a substrate for PLD. In a vast number of studies examining the hydrolysis of PtdIns(4,5,)P_2 the main $InsP_2$ isomers formed following agonist stimulation are Ins(1,4)P_2, Ins(1,3)P_2 and Ins(3,4)P_2 (see Berridge & Irvine, 1989) but not Ins(4,5)P_2 which would be the product of a PtdIns(4,5)P_2-PLD activity. Furthermore, PLD activity was also transient as judged by a number of criteria including the transient accumulation of PtdBuOH (Figure 3) and [^{3}H]-choline (Plevin & Wakelam, 1992).

Vasopressin-stimulated [^{3}H]-PtdBuOH accumulation was found to be subsequent to Ins(1,4,5)P_3 formation in A10 smooth muscle cells. This result is similar to that obtained in other peptide receptor systems such as endothelin-1 (ET-1)-

stimulated Rat-1 fibroblasts (MacNulty *et al.*, 1990) and bombesin-stimulated Swiss 3T3 fibroblasts (Cook *et al.*, 1990) and suggests that in this tissue PLC is activated before PLD. This argues in favour of a sequential pathway involving the activation of some type of intermediate. This is potentially PKC, since TPA stimulated the accumulation of PtdBuOH in the concentration-range consistent with its activation of PKC. Also both TPA- and vasopressin-stimulated [^{3}H]-PtdBuOH accumulation were completely inhibited by preincubation with the PKC inhibitor Ro-31-8220. These findings are consistent with results obtained in other tissues where down regulation of PKC by chronic phorbol ester pretreatment prevented subsequent activation of PLD (Cook & Wakelam, 1989; Martinson *et al.*, 1989; MacNulty *et al.*, 1990).

However, recent studies have shown that in some tissues agonist-stimulated PLD is only partially inhibited by PKC inhibitor pretreatment (Billah *et al.*, 1989; Liscovitch & Amsterdam, 1989; Cook *et al.*, 1991). This has implicated a PKC-independent component in the regulation of the PtdCh response, which has been suggested may involve a G-protein (Bocckino *et al.*, 1987; Martin & Michaelis, 1989). The construction of full inhibition curves for the effect of the PKC inhibitor, Ro-31-8220, upon agonist- and PMA-stimulated PLC activity yielded different IC_{50} values with the inhibitor being 15 fold more potent against the phorbol ester response (Figure 4). This result would suggest that agonist-stimulated PLD activity is not solely mediated by the prior activation of PKC and, since Ro-31-8220 is an ATP site inhibitor, PLD activation may involve the stimulation of additional kinases. However, Figure 5 demonstrates that pretreatment with Ro-31-8220 potentiates vasopressin-stimulated Ins(1,4,5)P_3 formation and thus the generation of DAG from PtdIn(1,4,5)P_2. Since in a number of previous studies it has been shown that the principal site of PKC-mediated negative feedback is at the level of the receptor/G-protein interface (e.g. Plevin *et al.*, 1990), it is reasonable to suggest that Ro-31-8220 may act to prolong PtdIns(4,5)P_2 hydrolysis in response to an agonist whose receptor is coupled to the effector PLC, by a G-protein. Indeed in Swiss 3T3 cells, vasopressin-stimulated [^{3}H]-InsP_3 accumulation is enhanced and prolonged in conditions where protein kinase C is inactive (Brown *et al.*, 1990). Although it is also possible that PKC may activate Ins-(1,4,5)P_3 kinase and/or phosphatase we have also shown previously that preincubation with Ro-31-8220 enhances vasopressin-stimulated [^{3}H]-IP accumulation in A10 VSMCs (Plevin & Wakelam, 1992). This argues against an effect on the rate of Ins(1,4,5)P_3 removal as the sole mechanism of action of PKC since this would have no effect on the accumulation of total [^{3}H]-IP. Thus, it remains possible that the maintenance of a PtdIns(4,5,)P_2-derived DAG signal may activate a form of PKC which requires a greater concentration of Ro-31-8220 to be fully inhibited.

In vascular smooth muscle cells, calcium influx is important in the regulation of a number of intracellular events (Zschauer *et al.*, 1987; Ruegg *et al.*, 1989). The results in this study suggest a partial involvement of extracellular calcium in the regulation of vasopressin-stimulated PLD activity in A10 smooth muscle cells. Buffering with EGTA resulted in a small but significant reduction (approximately 30%) in the vasopressin-stimulated response, whilst pretreatment with a series of calcium channel antagonists was without effect. This includes the L-type voltage dependent calcium antagonists, nifedipine and verapamil and also SK&F 96365 which it has been proposed inhibits receptor mediated calcium influx (Merrit *et al.*, 1990). This compound was only effective at high micromolar concentrations where it acted in a non-specific manner, being equally effective against K^+-stimulated PLD activity. Indeed the original studies by Merrit *et al.* (1990) have suggested that some smooth muscle cells may contain receptor-operated calcium channels insensitive to this type of compound.

The largely calcium-independent nature of vasopressin-stimulated PLD activity suggests that agonist activation of PKC (and thus PLD activity) can be almost fully achieved at resting levels of intracellular Ca^{2+}. This was confirmed by analysing the dose-response relationship for Ro-31-8220 inhibition of vasopressin-stimulated [^{3}H]-PtdBuOH accumulation in calcium-free conditions. This parameter was not significantly affected, which suggests that the sensitivity of agonist-stimulated [^{3}H]-PtdBuOH accumulation to inhibition by Ro-31-8220 is not changed in the absence of extracellular calcium. Furthermore, the finding that A23187-stimulated PLD activity is completely abolished by PKC inhibitor pretreatment once again emphasizes the central role PKC may play in the regulation of PLD in this cell. These findings are consistent with that obtained for ATP-stimulated endothelial cells (Martin & Michaelis, 1989), vasopressin-stimulated hepatocytes (Bocckino *et al.*, 1987) and with a recent report that showed no effect of chelation with 10 mM EGTA upon PLD activity in A10 smooth muscle cells in culture (Welsh *et al.*, 1990). However, in other tissues, agonist-stimulated PLD activity is reduced substantially upon extracellular calcium removal (Pai *et al.*, 1988; Lassegue *et al.*, 1991; Wakelam *et al.*, 1991). In these systems Ca^{2+} influx may also directly regulate PLD activity in addition to modifying PKC activity.

In this study we have examined the characteristics of vasopressin-stimulated PtdIns(4,5)P_2 and phosphatidylcholine hydrolysis in A10 vascular smooth muscle cells. The results indicate that the hydrolysis of both lipids are regulated by interaction with the same receptor subtype. Vasopressin stimulation of PLD is achieved through a sequential pathway, subsequent to the initial PtdIns(4,5)P_2 hydrolysis and involving the intermediate activation of PKC. Calcium entry appears to have a small but significant regulatory role in vasopressin-stimulated PLD activity in this tissue.

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