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Phorbol ester inhibits arginine vasopressin activation of phospholipase C and promotes contraction of, and prostaglandin production by, cultured mesangial cells

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We have previously shown that arginine vasopressin (AVP) causes a rapid (5–10 min) contractile response in cultured mesangial cells plated onto slippery substrata such as poly(hydroxyethyl methacrylate)-coated dishes. This contraction is associated with an increase in the levels of inositol trisphosphate ($InsP_3$), diacylglycerol and prostaglandin E_2 (PGE_2). We now report that agents which are known to activate protein kinase C, i.e. phorbol 12-myristate 13-acetate (PMA) and oleolylacetylglycerol (OAG), also contract mesangial cells; however, the contractile response is slow to develop (15–30 min). The inactive phorbol ester, 4α-phorbol 12,13-didecanoate, did not elicit contraction. PMA and OAG did not increase $InsP_3$ release in mesangial cells. However, pretreatment of mesangial cells with PMA inhibited the formation of $InsP_3$. This inhibition could not be explained by a reduction in AVP binding since PMA treatment did not influence the number or affinity of [^{3}H]AVP binding sites in intact cells. PMA alone stimulated PGE_2 production in mesangial cells to a degree similar to AVP. Contrary to what was seen with $InsP_3$, pretreatment of cells with PMA before AVP had an additive effect on arachidonic acid release and PGE_2 production. Thus, there is an apparent dissociation of phospholipase C activity from that of phospholipase A_2.

INTRODUCTION

Cellular signalling mechanisms utilizing receptors linked to Ca mobilization are associated with rapid stimulation of phosphoinositide metabolism. Increased phosphoinositide metabolism is, in turn, characterized by inositol trisphosphate ($InsP_3$) formation and increased levels of diacylglycerol. $InsP_3$ is thought to cause release of Ca from intracellular stores, leading to a transient rise of cytosolic Ca. Diacylglycerol has been associated with stimulation of protein kinase C, an enzyme which phosphorylates serine and threonine residues (Nishizuka, 1984). PMA stimulates protein kinase C, and this compound has been used extensively to study cellular effects of protein kinase C action (Castagna *et al.*, 1982). These effects include phosphorylation of lipomodulin (Hirata *et al.*, 1984), an endogenous phospholipase-regulating protein, and myosin light chain (Nishikawa *et al.*, 1984). This phosphorylation of myosin light chain occurs at a different site from that phosphorylated by myosin light chain kinase. An additional regulatory function of protein kinase C may include down-regulation of $InsP_3$ formation (Brock *et al.*, 1985). It may also play a role in long-term (tonic) stimulus–response coupling mechanisms (Kojima *et al.*, 1983). Several studies of smooth-muscle preparations suggest a role for protein kinase C in the sustained phase of contraction (Danthuluri & Deth, 1984; Rasmussen *et al.*, 1984; Chatterjee & Tejeda, 1986) which persists beyond the more transient elevation of cytosolic Ca (Morgan & Morgan, 1982; Aksoy *et al.*, 1983) and myosin light chain phosphorylation (Silver & Stull, 1982; Aksoy *et al.*, 1983).

The purpose of these studies was to examine the effects of phorbol 12-myristate 13-acetate (PMA) and oleolylacetylglycerol (OAG) on phosphoinositide metabolism and contraction in cultured glomerular mesangial cells. These cells are contractile cells of the renal glomerulus where long-term (tonic) regulation of vascular tone plays an important role in determining glomerular filtration rate. Previous studies of mesangial cells indicate that PMA inhibits angiotensin II-stimulated $InsP_3$ release (Pfeilschifter, 1986). However, the present studies emphasize that, in spite of suppression of $InsP_3$ release, PMA treatment induces contraction of mesangial cells.

MATERIALS AND METHODS

Contraction studies

Mesangial cells were isolated and grown in homogeneous culture as described previously (Troyer *et al.*, 1985). For the contraction studies, cells were plated onto polyHEMA-coated tissue-culture dishes (Venkatachalam & Kreisberg, 1985). Cells plated onto polyHEMA are less adherent and respond to arginine vasopressin (AVP) by isotonically contracting (i.e. cell rounding) (Venkatachalam & Kreisberg, 1985). Cells were exposed to vasopressin (10 nM), PMA (16 nM–1.6 μM), the inactive phorbol ester 4α-phorbol 12,13-didecanoate (PDD) (1.5 μM), and OAG (5–50 μg/ml) in RPMI 1640 tissue-culture medium. The cells were

Abbreviations used: AVP, arginine vasopressin; polyHEMA, poly(hydroxyethyl methacrylate); PGE_2, prostaglandin E_2; PMA, phorbol 12-myristate 13-acetate; OAG, oleoylacetylglycerol; PDD, 4α-phorbol 12,13-didecanoate; RPMI, Roswell Park Memorial Institute Medium; $InsP_3$, inositol trisphosphate.

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observed continuously for contraction under a Zeiss inverted phase-contrast microscope (Dexter Instruments, San Antonio, TX, U.S.A.) equipped with an air-stream stage incubator (Nicholson Precision Instruments, Bethesda, MD, U.S.A.) to maintain the temperature at 37 °C. The stage was housed in a Plexiglass box through which a 19:1 air/CO_2 mixture was circulated. Contraction was recorded photographically. Cells were considered to be contracted when they became highly refractile with rounded cell bodies (Venkatachalam & Kreisberg, 1985). At least 10 cells per dish were observed for a contractile response. Mesangial cells were tested for contraction between the 30th and 60th passages in culture.

Phospholipid studies

For studies of $InsP_3$ release, cells were labelled for 48 h with 5 μCi of [^{3}H]inositol per 60 mm dish containing 2 ml of culture medium. The final results are expressed as d.p.m./dish since the protein content/dish did not vary significantly within a given passage. The water-soluble inositol phosphates were separated on AG1X8 Dowex anion-exchange columns eluted with ammonium formate and formic acid as described previously (Troyer *et al.*, 1985). We have used the term $InsP_3$ rather than inositol 1,4,5-trisphosphate in reference to the inositol phosphates eluted from Dowex AG1X8 columns with 0.1 M-formic acid/1.0 M-ammonium formate. The existence of at least one isomer in addition to inositol 1,4,5-trisphosphate in such eluates was demonstrated by Batty *et al.* (1985). Further studies will be necessary to elucidate the possible existence and significance of isomers of inositol 1,4,5-trisphosphate in our system. All radiolabelling experiments were conducted using RPMI as the experimental medium.

[^{3}H]AVP binding

Incubation buffer for the binding assay was RPMI with 1% bovine serum albumin, 1 mM-phenylmethanesulphonylfluoride, 100 μg of bacitracin/ml, 1.4–42 ng of [^{3}H]AVP/ml (10 Ci/mmol) in the presence and absence of 10^{-5} M unlabelled AVP in a final assay volume of 0.5 ml. Incubations were conducted with triplicate determinations of specific and non-specific binding at each dose level of AVP in 9.2 cm^2 wells [$(1.5–2.2) \times 10^6$ cells/well]. Cells were washed twice with RPMI before initiating binding assays. Cells were incubated with PMA (160 nM) for 5 min at 37 °C and cooled for 10 min at 4 °C before initiating binding studies. AVP binding at 4 °C reached steady state by 20 min and was stable for an additional 20 min. Incubations were terminated by washing twice with 0.2 M-sodium phosphate-buffered saline, pH 7.4. Cells were digested with 500 μl of 0.1 M-NaOH and a 400 μl portion was removed for counting with Biofluor (New England Nuclear, Boston, MA, U.S.A.) in a liquid-scintillation counter. Results were calculated by regression analysis employing a Hewlett–Packard 11C calculator. The results demonstrated a single class of binding sites.

Measurements of prostaglandin E_2 (PGE_2), fatty acids, and diacylglycerol

PGE_2 was assayed on media collected from cells in experiments designed in an analogous manner to study of phospholipid metabolism. Anti-sera for PGE_2 radioimmunoassay was from Institut Pasteur (Paris, France) and cross-reactivity has been described previously (Dray *et al.*, 1975).

Agonist-stimulated mobilization of free arachidonic acid was studied in cells labelled for 18 h with 3 μCi of [^{3}H]arachidonic acid/dish in 2 ml of culture medium. Cells were washed, then incubated with RPMI containing 0.5% fatty-acid-free bovine serum albumin, followed by extraction by the method of Bligh & Dyer (1959). Neutral lipids were separated on silica-gel G plates using hexane/diethyl ether/acetic acid (60:40:1, by vol.).

Materials

[^{3}H]Inositol and [^{3}H]arachidonic acid were purchased from New England Nuclear. [^{3}H]AVP was purchased from Amersham. RPMI 1640 culture medium was purchased from Irvine Scientific. Fetal-calf serum was obtained from Grand Island Biological Co. [8-Arg]-vasopressin was purchased from Vega Biochemicals or Boehringer–Mannheim.

RESULTS

Contraction

Mesangial cells grown on polyHEMA contract within 5–10 min in response to AVP, and this contractile responsiveness was shown to be dependent upon extracellular Ca (Venkatachalam & Kreisberg, 1985). PMA and OAG also cause mesangial cell contraction (Figs. 1 and 2 and Table 1). However, the contraction took as long as 30 min to maximize. The contractile response was reversible and, unlike AVP, independent of extracellular Ca at higher doses of PMA (1.6 μM and 160 nM). Significant contraction (75% of the cells contracting) was seen with 160 nM-PMA and virtually all the cells contracted with 1.6 μM-PMA (Table 1). At a concn. of 16 nM, 30–50% of the cells contracted. However, addition of a low non-contractile dose of A23187 (0.03 μg/ml) caused 75–100% of the cells to contract in the presence of 16 nM-PMA. OAG (25 μg/ml) caused 75–100% of the cells to contract (Table 1), while lower concentrations (5 μg/ml) contracted 30–50% of the cells. The inactive phorbol ester, PDD, did not contract the cells (Table 1).

Phospholipid metabolism

Vasopressin treatment of mesangial cells increases the formation of $InsP_3$ (Troyer *et al.*, 1985). Vasopressin also increases intracellular free Ca as measured by the fluorescent Ca chelators quin2 and fura2 (Bonventre *et al.*, 1986). Neither PMA nor OAG alone, at concentrations which stimulate mesangial contraction, had a significant effect on $InsP_3$ release (Table 2 and Fig. 3). However, also shown in Fig. 3, PMA pretreatment markedly reduced the amount of $InsP_3$ released following AVP stimulation. OAG pretreatment also reduced $InsP_3$ release but to a lesser extent than PMA. As shown in Fig. 4, half-maximal inhibition was achieved at approx. 10 nM. When we increased the concentrations of AVP (100 nM) and PMA (160 nM) to those used in the prostaglandin study (see Table 4), this resulted in less inhibition of AVP-stimulated $InsP_3$ release. [Results (d.p.m./dish $\pm$ S.D.): AVP, 1680 ± 62; AVP+PMA, 1192 ± 92; PMA, 507 ± 28; control, 373 ± 41]. The inactive phorbol ester PDD (1.5 μM) did not inhibit $InsP_3$ release by vasopressin (results not shown).

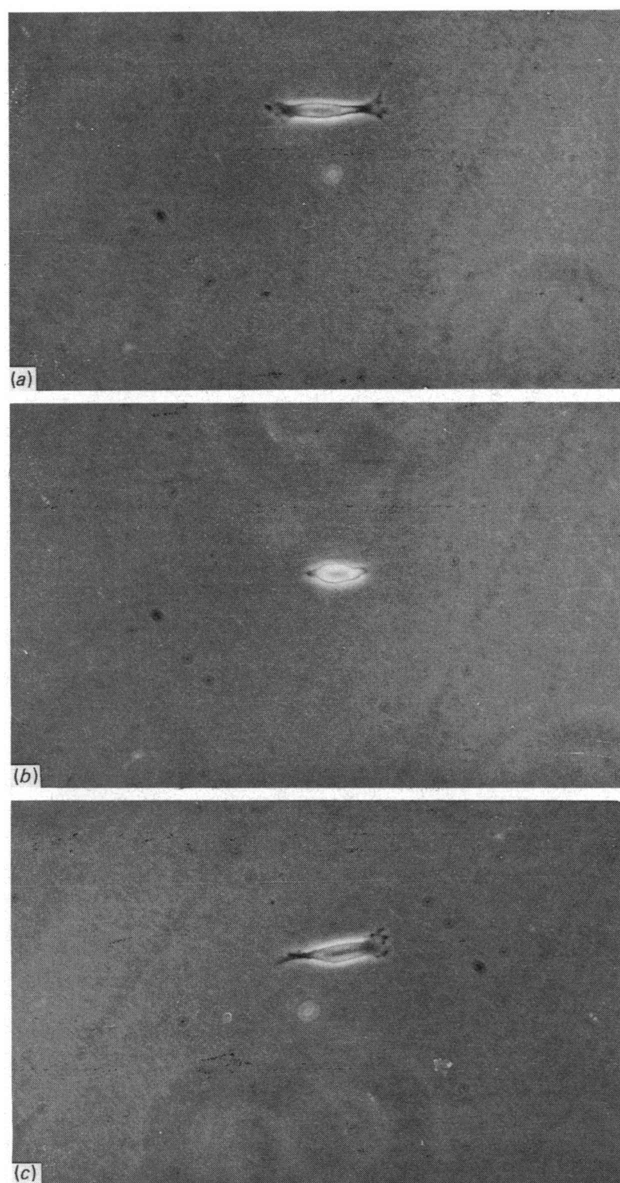

Fig. 1. Phase-contrast photomicrograph of a mesangial cell plated onto a polyHEMA-coated dish

(*a*) Before treatment; (*b*) 15 min after exposure to 16 nM-PMA. Notice the cell has isotonically contracted (cell rounding). (*c*) 15 min later, after replacement of the PMA-containing medium with fresh RPMI, the cell begins to relax. Magnification 205×.

Effect of PMA on [^{3}H]arginine vasopressin binding

To assess whether PMA was exerting an inhibitory effect on AVP-induced $InsP_3$ release by modulating vasopressin binding, we examined [^{3}H]AVP binding to intact mesangial cells which had been incubated in the presence of PMA (160 nM) for 5 min before the binding assay. Neither binding affinity nor receptor density was affected by PMA (control, K_d 17±2 nM, $B_{max.}$ 647±147 fmol/10^6 cells; PMA-treated, K_d 25±7.8 nM, $B_{max.}$ 675±173 fmol/10^6 cells, $n = 4$). We also examined longer (15 min) incubations with PMA (160 nM) and found similar results (results not shown).

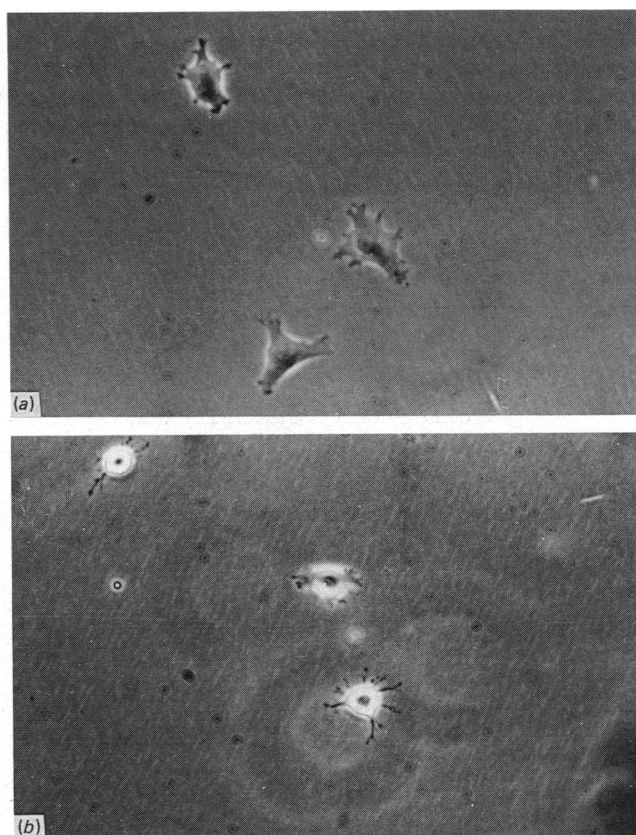

Fig. 2. Phase-contrast photomicrograph of mesangial cells plated onto polyHEMA

(*a*) Before treatment; (*b*) 30 min after addition of 160 nM-PMA. The cells have contracted. Magnification 205×.

Table 1. Effect of phorbol esters on mesangial cell contraction

Mesangial cells were plated onto polyHEMA-coated Petri dishes (1:250 alcoholic dilution of polyHEMA). For contraction studies, cells were incubated in RPMI 1640 tissue-culture medium. Numbers in parentheses indicate numbers of dishes observed. The results given represent a semi-quantitative evaluation of the proportion of contracting cells. Only cells which displayed morphological features as shown in Figs. 1 and 2 were considered to be contracted.

Agent	Contractile response (% of cells contracting)
PMA (1.6 μM)	100 (6)
PMA (160 nM)	50–75 (6)
PMA (16 nM)	30–50 (6)
PMA (16 nM)+A23187 (0.03 μg/ml)	75–100 (3)
PDD (1.5 μM)	0 (3)
OAG (25 μg/ml)	75–100 (6)
OAG (5 μg/ml)	30–50 (6)

Unesterified fatty acids, diglyceride and prostaglandin production

Evidence supporting increased mobilization of [^{3}H]-arachidonic acid is shown in Table 3. These cells were treated with PMA and AVP, and the two agents showed

Table 2. Inositol trisphosphate formation during treatment of cells with PMA, OAG, and AVP

Cells prelabelled for 48 h with [^{3}H]inositol were rinsed and then exposed to the agents for 20 min. Values shown are means of d.p.m./dish ± S.D. (n = 4).

Treatment	$InsP_3$ formation (d.p.m./dish)
PMA (100 nM)	146 ± 50
OAG (63 × 10^3 nM)	100 ± 11
Control	106 ± 7
AVP (10 nM)	1034 ± 32

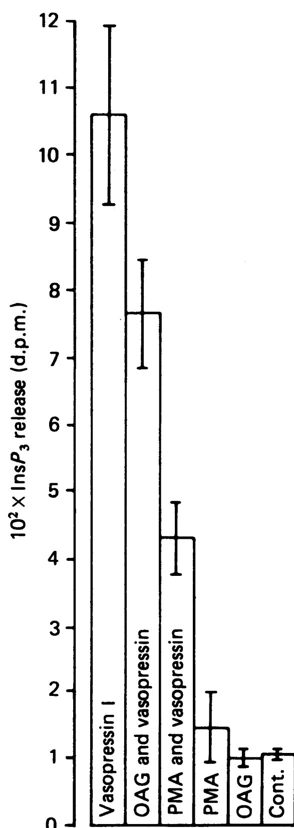

Fig. 3. Effects of PMA and OAG on vasopressin-stimulated $InsP_3$ release

Cells were labelled for 48 h with [^{3}H]inositol, then exposed to OAG, PMA, or experimental medium for 5 min. The incubation was then continued for 20 min by adding AVP to one group of cells. (OAG, 25 μg/ml; PMA, 100 nM; AVP, 10 nM). Values shown are means ± S.D. (n = 4). Abbreviation: Cont, control.

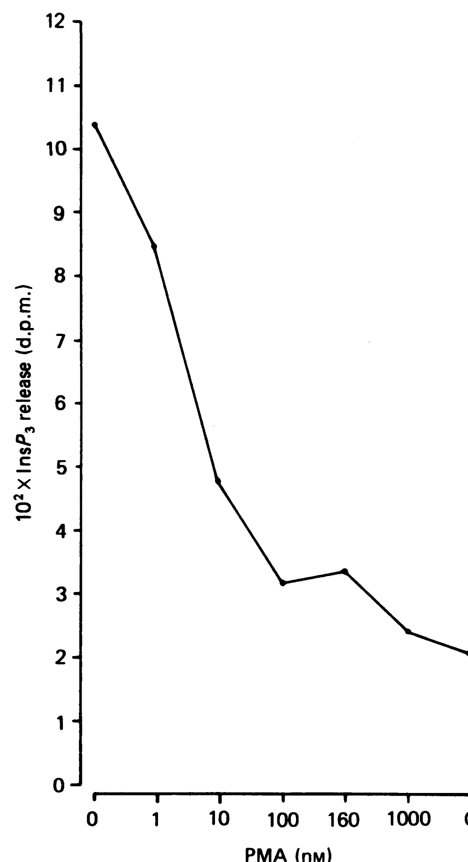

Fig. 4. Dose–response effect of PMA on $InsP_3$ release

Cells were labelled and incubated as for Fig. 4 except that varying concentrations of PMA were used as indicated. Values shown are means of four determinations at each dose.

Table 3. Formation of unesterified fatty acids

Cells prelabelled with [^{3}H]arachidonic acid were washed and then incubated in serum-free RPMI for 90 min. Incubations were then continued and portions of the medium taken for fatty acid extraction at the indicated times. Values show mean d.p.m. ± S.D. (n = 4).

Treatment	Free fatty acid release (d.p.m.)	
Time ...	15 min	45 min
PMA + AVP	44144 ± 3631	73740 ± 12389
PMA	18732 ± 2917	62709 ± 6830
AVP	19194 ± 2739	24866 ± 3979
Control	5215 ± 1164	15013 ± 3300

an additive effect on free fatty acid release. We also studied the influence of PMA on prostaglandin production. We have previously shown that AVP stimulates PGE_2 production in mesangial cells (Troyer *et al.*, 1985). Table 4 shows that PMA stimulates PGE_2 production by as much as 300 % above basal production. Also shown in Table 4 are the effects of increasing doses of PMA and vasopressin, and the additive effects of simultaneous treatment with both PMA and vasopressin. This suggested that either PMA mobilizes a separate or additional pool of arachidonic acid or increases the efficiency of conversion of arachidonic acid to prostaglandins.

DISCUSSION

The results of this study show that substances which activate protein kinase C (i.e. PMA and OAG) cause a reversible contractile response which is slow in onset and similar to that observed by Rasmussen *et al.* (1984) in

Table 4. PGE_2 production by mesangial cells

Values show means ± S.E.M. of PGE_2 production in ng/15 min incubation per ml. $^*P < 0.01$ comparing AVP + PMA with either AVP alone or PMA alone.

Treatment	PGE_2 production (ng/15 min incubation per ml)	(*n*)
Control	0.6 ± 0.1	(11)
10^{-7} M-AVP + 160 nM-PMA	4.6 ± 0.3*	(6)
10^{-7} M-AVP	3.0 ± 0.3	(9)
160 nM-PMA	2.6 ± 0.04	(15)
10^{-8} M-AVP + 16 nM-PMA	3.6 ± 0.3*	(6)
10^{-8} M-AVP	2.1 ± 0.3	(3)
16 nM-PMA	2.5 ± 0.4	(3)

phorbol ester-treated rabbit vascular smooth muscle. PMA also seems to inhibit phospholipase C activation since AVP-induced $InsP_3$ release is reduced.

Vasopressin causes release of $InsP_3$, increased formation of diacylglycerol, elevation of intracellular free Ca (Troyer *et al.*, 1985; Bonventre *et al.*, 1986) and contraction of cultured mesangial cells (Venkatachalam & Kreisberg, 1985). The increase in diacylglycerol which accompanies contraction could, in combination with Ca, activate another second messenger, protein kinase C. Thus, it has been suggested that interactions between the inositol lipid and protein kinase C pathways may occur. Rasmussen and co-workers suggest, based on studies of angiotensin II-induced aldosterone secretion by adrenal glomerulosa cells, that angiotensin II stimulates breakdown of phosphatidylinositol 4,5-bisphosphate which leads to generation of two second messengers; $InsP_3$ which releases Ca from intracellular non-mitochondrial stores causing the rapid angiotensin effect, and diglyceride which activates protein kinase C contributing to the sustained phase of the angiotensin II response (Rasmussen, 1986). An interaction between Ca and PMA action is suggested by our finding that PMA (16 nM)-induced mesangial cell contraction was augmented by the addition of A23187 (Table 1). Thus, maximum contraction can be achieved with a low dose of PMA (16 nM) by adding a non-contracting amount of the Ca ionophore A23187 (Table 1). This suggests that the action of PMA to induce contraction does require some low level of Ca, possibly from an extracellular source.

One aspect of this long-term activation may involve negative feedback of the diacylglycerol/PMA arm of the pathway on phospholipase C generation of inositol trisphosphate. In support of this hypothesis, PMA (which substitutes for diacylglycerol to activate protein kinase C) inhibits vasopressin-induced $InsP_3$ release in mesangial cells and causes a slow contractile response.

Brock *et al.* (1985) and Pfeilschifter (1986) also found that PMA and OAG prevented angiotensin II-induced $InsP_3$ release and Ca elevation in cultured aortic smooth-muscle cells and mesangial cells. However, contraction was not examined during these studies. Our studies confirm that PMA suppresses hormone-induced $InsP_3$ release, but we also show that PMA produces contraction of mesangial cells. These results support the hypothesis advanced by Rasmussen (1986) that protein kinase C may be involved in sustained contraction of smooth-muscle cells. It has been shown that activation of protein kinase C in platelets results in phosphorylation and activation of an inositol trisphosphate 5′-phosphomonoesterase which breaks down $InsP_3$ (Connolly *et al.*, 1986). This suggests a mechanism by which PMA could reduce the level of $InsP_3$ in hormone-stimulated cells.

Finally, we found an additive effect of PMA and vasopressin on prostaglandin production. This stands in contrast with the effects of PMA on release of inositol phosphates and suggests that phospholipase C activation occurs independently of prostaglandin production as we suggested earlier (Troyer *et al.*, 1985). It also supports the existence of separate coupling mechanisms for prostaglandin production (i.e. phospholipase A_2 activation) and phospholipase C activation. However, we note that our results did not show complete suppression of vasopressin-stimulated $InsP_3$ release by vasopressin. It is therefore possible that cellular Ca levels are higher in cells treated with PMA plus vasopressin than in those receiving PMA alone. This, combined with the effects of PMA on phospholipase A_2, could result in a synergistic increase of unesterified fatty acids when both agents are present.

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