Sustained phospholipase D activation in response to angiotensin II but not carbachol in bovine adrenal glomerulosa cells

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We have demonstrated previously that in bovine adrenal glomerulosa cells, phospholipase D (PLD) activity can indirectly result in the generation of *sn*-1,2-diacylglycerol (DAG) through its production of phosphatidic acid (PA) and the subsequent action of PA phosphohydrolase. Furthermore, the PLD-generated DAG can trigger aldosterone secretion. Therefore, we characterized PLD activation by two agonists, angiotensin II (Ang II) and carbachol, to determine if the activity of the enzyme might underlie sustained aldosterone secretion. We determined that Ang II-induced PLD activation occurred via the angiotensin-1 receptor (AT_1), and that a specific AT_1 antagonist, losartan, inhibited this activation, whereas the same concentration of the AT_2-specific antagonist, PD 123319, had no effect. Ang II activated PLD with a dose dependence similar to that observed for aldosterone secretion, with slight increases in activity induced by 0.1 nM Ang II and maximal activation at 10 nM. We also found that Ang II induced a sustained activation of PLD, but that the effect of carbachol, a stable analogue of acetylcholine, was transient; PLD activity increased within 5 min of exposure to carbachol but then ceased by 15 min. Higher carbachol concentrations were also unable to sustain PLD activation. These results suggest that the Ang II-elicited activation of PLD is associated with a sustained increase in aldosterone secretion from glomerulosa cells and further provide the first evidence, to our knowledge, of differences in the kinetics of PLD activation in response to two physiologically relevant agonists. Finally, we speculate that this disparity correlates with different functional responses induced by the two agents.

INTRODUCTION

Upon binding to its receptor in bovine adrenal glomerulosa cells, angiotensin II (Ang II) activates a phosphoinositide-specific phospholipase C (PI-PLC) which hydrolyses Ins(4,5)P_2 to generate the second messengers, Ins(1,4,5)P_3 and *sn*-1,2-diacylglycerol (DAG) [1,2]. An increase in Ins(1,4,5)P_3 content is thought to initiate aldosterone secretion by eliciting a transient increase in the cytosolic Ca^{2+} concentration [3] which, in turn, activates Ca^{2+}/calmodulin-dependent protein kinases [4]. On the other hand, DAG functions to stimulate protein kinase C (PKC), an effector enzyme that is thought to play a role in the regulation of aldosterone secretion from bovine adrenal glomerulosa cells. In addition to the elevation of DAG levels, Ang II also increases and maintains Ca^{2+} influx, which is essential for sustained aldosterone secretion (reviewed in [3]). Changes in Ca^{2+} influx are thought to regulate PKC activity in the presence of elevated membrane DAG content; indirect evidence for this concept is provided by studies which showed that a full aldosterone secretory response can be produced pharmacologically only by a combination of agents, one of which provides the DAG signal and the other which increases Ca^{2+} influx ([5], and reviewed in [3]). Thus it has been postulated that the Ca^{2+} influx requirement is the result of the Ca^{2+} sensitivity of PKC (i.e. a conventional isoenzyme). Recently, direct evidence for the Ca^{2+} sensitivity of PKC in bovine glomerulosa cells has been obtained: in digitonin-permeabilized cells, the ability of Ang II-activated PKC to phosphorylate a specific peptide substrate of PKC is dependent on the Ca^{2+} concentration [6]. Thus it has been hypothesized that during the sustained phase of the response, a new three-component plasma membrane transducer is generated consisting of DAG, Ca^{2+} cycling, and PKC, and that this system underlies the sustained phase of the Ang II-elicited increase in aldosterone secretion [7].

Another agent that activates PI-PLC in adrenal glomerulosa cells is the stable analogue of acetylcholine, carbachol, with the concomitant production of Ins(1,4,5)P_3 [8] and DAG [9]. Like Ang II, carbachol also increases Ca^{2+} influx, although to a lesser extent than Ang II [8]. Unlike Ang II, which induces a monotonic increase in the aldosterone secretory response, carbachol elicits a biphasic increase in aldosterone secretion in freshly-isolated glomerulosa cells, with a peak increase of six-fold over the basal secretory rate at approx. 15 min, followed by a fall to a sustained plateau which remained at a level of about four-fold over the control rate for at least 30–40 min [8]. Enhancement of Ca^{2+} influx using the Ca^{2+} channel agonist, Bay K8644, to a rate comparable with that elicited by Ang II resulted in a monophasic, sustained carbachol-induced steroidogenic response similar to that stimulated by Ang II [8]. Thus it was proposed that the biphasic secretory response to carbachol was the result of a lesser hormone-elicited increase in Ca^{2+} influx [8]. However, we also found that carbachol was less effective than Ang II at inducing increases in DAG content and [^{3}H]myristate-containing DAG [9].

While DAG can be generated directly by the action of phospholipase C on phospholipids, this compound can also be

Abbreviations used: ACTH, adrenocorticotrophic hormone; Ang II, angiotensin II; AT, angiotensin receptor; DAG, *sn*-1,2-diacylglycerol; KRB^+, bicarbonate-buffered Krebs–Ringer solution containing 2.5 mM sodium acetate; PA, phosphatidic acid; PEt, phosphatidylethanol; PI-PLC, phosphoinositide-specific phospholipase C; PKC, protein kinase C; PLD, phospholipase D.

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produced by the combined activity of phospholipase D (PLD) and PA phosphohydrolase [10]. Thus, PLD forms PA which can be dephosphorylated by PA phosphohydrolase to yield DAG. DAG can, in turn, be converted to PA by the action of DAG kinase or metabolized to monoacylglycerol by DAG lipase [11]. PLD also has the unique characteristic of effecting ethanolysis of phospholipids in the presence of a small amount of ethanol to yield the novel phospholipid phosphatidylethanol (PEt) [12]. We have demonstrated previously that Ang II can increase radioactively labelled PEt levels in [^{3}H]oleate-labelled glomerulosa cells at a single (30 min) time point, indicating that this hormone activates PLD [5]. Furthermore, using exogenous bacterial PLD as a tool, we demonstrated that PLD-generated PA can be converted to DAG by endogenous PA phosphohydrolase and elicit secretion [5]. While exogenous PLD alone has only a modest effect on the aldosterone secretory response, addition of the Ca^{2+}-channel angonist, Bay K8644, to provide the other Ang II-generated signal, Ca^{2+} influx, results in secretion that is comparable with that elicited by Ang II [5]. These data suggested a potential role for PLD in sustained Ang II-induced steroidogenesis.

Consistent with this suggestion, we found that carbachol is less effective than Ang II in inducing PLD activation, measured, at 30 min, as an increase in [^{3}H]PEt levels. Since PLD is known to hydrolyse phosphatidylcholine [13–16] and [^{3}H]myristate is incorporated primarily into this phospholipid [17], we proposed that myristate-containing DAG may be generated by PLD and PA phosphohydrolase. Thus the inefficient activation of PLD by carbachol could explain the inefficient increases in DAG content and [^{3}H]myristate-containing DAG triggered by this hormone, as well as, perhaps, the biphasic carbachol-elicited aldosterone secretory response. In view of the potential role of PLD in sustained agonist-induced aldosterone secretion, we sought to characterize further PLD activation elicited by Ang II and by carbachol.

These data were presented, in part, at the International Congress of Endocrinology, San Francisco, U.S.A. in June 1996.

MATERIALS AND METHODS

Bovine glomerulosa cell preparation and culture

Bovine glomerulosa cells were isolated as described previously [17]. Briefly, glomerulosa cell slices were prepared from near-term fetal adrenal glands obtained from a local slaughterhouse, and glomerulosa cells were dispersed from collagenase-digested slices by mechanical agitation. The freshly-isolated glomerulosa cells were cultured overnight in Falcon Primaria dishes (Becton Dickinson Labware, Lincoln Park, NJ, U.S.A.) in a Dulbecco's modified Eagle's medium/Ham's F12 medium (1:1, v/v) containing: 10% (v/v) horse serum, 2% (v/v) fetal bovine serum, 100 μM ascorbate, 1.2 μM α-tocopherol, 0.05 μM Na_2SeO_3, 50 μM butylated hydroxyanisole, 5 μM metyrapone, 100 units/ml penicillin, 100 μg/ml streptomycin, 25–30 μg/ml gentamycin and 0.25 μg/ml amphotericin B, essentially as described previously [17]. After replacement of the serum-containing medium with serum-free medium [containing 0.2% (w/v) BSA and 5 μCi/ml [^{3}H]oleic acid], the cells were incubated for an additional 20–24 h before use. In some experiments, fetal bovine serum was increased to 4% (v/v) to promote attachment and survival of the cells.

PLD activity assay

The PLD activity assay was performed essentially as described in [5], cultured primary adrenal glomerulosa cells were radioactively labelled in serum-free medium containing 5 μCi/ml [^{3}H]oleic acid for 20–24 h. ([^{3}H]Oleate was chosen to monitor the formation of PA and PEt because this fatty acid is a major constituent and, thus, labels the phosphatidyl moiety of all phospholipids. Therefore, using this fatty acid, PLD activity can be monitored regardless of the identity of the phospholipid which serves as the substrate of the enzyme.) The medium was aspirated, the cells were rinsed once with bicarbonate-buffered Kreb's–Ringer solution [120 mM NaCl/24.9 mM $NaHCO_3$/3.5 mM KCl/1.2 mM $MgSO_4$/1.2 mM NaH_2PO_4/1.25 mM $CaCl_2$/0.1% (w/v) dextrose/0.2% (w/v) BSA] containing 2.5 mM sodium acetate (KRB^+) and were preincubated for 30 min in KRB^+. The cells were then stimulated with the appropriate agonists and incubated for the indicated time periods in the presence of 0.5% ethanol, as described in the Figure legends. All incubations were performed in a CO_2/air (19:1) incubator. The reactions were terminated and the cells solubilized by the addition of 0.2% (w/v) SDS containing 5 mM EDTA and were extracted for 1–2 h with ice-cold chloroform/methanol/acetic acid (1:2:0.04, by vol.). Additional chloroform and 0.2 M NaCl were added to generate two phases, and the lower phases were collected and dried under a flow of N_2. The samples were resuspended in chloroform/methanol (2:1, v/v) containing 25–50 μg PEt and PA per sample and were applied on to heat-activated silica gel 60 TLC plates (0.25-mm thickness, aluminum-backed with a concentrating zone). The plates were developed in the upper phase of a solvent system of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (13:2:3:10, by vol.) and separated products were revealed with iodine vapour and by autoradiography using En^{3}Hance. Spots corresponding to PA and PEt, identified by comigration with authentic standards, were excised, placed in scintillation fluid and quantified by liquid scintillation spectrometry.

Measurement of DAG content using the DAG kinase assay

Briefly, cells were incubated in KRB^+ in the presence and absence of 10 nM Ang II and 100 μM propranolol or 1% ethanol for 15–30 min and the reactions were terminated by the addition of 0.2% (w/v) SDS. The lipids were then extracted into chloroform/methanol (2:1, v/v) as described previously [17], and the DAG content was determined using a bacterial DAG kinase to phosphorylate DAG in the presence of [γ-^{32}P]ATP to yield [^{32}P]PA. [^{32}P]PA was then separated by TLC and quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). This method has been described previously for the measurement of cellular DAG content by Preiss et al. [18] and was modified in our laboratory [2].

Aldosterone secretion assay

Duplicate or triplicate wells of cultured glomerulosa cells were washed 2–3 times with KRB^+ and incubated for 2 h in a 37 °C CO_2/air (19:1) incubator. Supernatants were removed and the cells treated with the appropriate agonists in the presence and absence of ethanol. Note that for 10 mM potassium stimulation, the sodium concentration of the KRB^+ was adjusted to maintain equivalent osmolarity, as described in [19]. After an additional 2-h incubation at 37 °C in CO_2/air (19:1), the supernatants were again removed and the cells were solubilized in 0.3 M NaOH for determination of protein content. Supernatants were stored frozen until assay of aldosterone levels, using a commercially available radioimmunoassay (Diagnostic Products, Los Angeles, CA, U.S.A.).

Protein assay

Protein amounts in small aliquots of the SDS extracts were determined using the BioRad (Hercules, CA, U.S.A.) or the micro-bicinchoninic acid (BCA) (Pierce, Rockford, IL, U.S.A.) assay, with BSA as standard.

Statistical analysis

The significance of differences between mean values was determined using analysis of variance (ANOVA), as performed by the program Instat (GraphPad Software, San Diego, CA, U.S.A.).

RESULTS

Initially, to determine whether PLD activation could play a role in regulating Ang II-induced aldosterone secretion, it was important to ascertain the Ang II receptor subtype involved in hormone-induced PLD activation. The angiotensin receptor (AT)-1 (AT_1) has been reported to mediate the steroidogenic effects of Ang II in glomerulosa cells; the function of the AT_2 receptor is largely unknown (reviewed in [20]). We used the AT_1-specific antagonist losartan (DuP 753) and the AT_2-specific antagonist PD 123319 to determine through which receptor subtype PLD was activated. The [^{3}H]oleate-labelled primary glomerulosa cells were stimulated for 30 min in the presence of 0.5% (v/v) ethanol and phospholipids were extracted and separated by TLC as described in the Materials and methods section. As shown in Table 1, losartan at a dose of 10 μM inhibited Ang II-induced PA and PEt formation by 47% and 67% respectively, while exerting little or no effect on control PLD activity. At the same concentration, PD 123319, on the other hand, had little or no effect on either Ang II-stimulated or basal PLD activity (Table 1). These results indicate that PLD activation in response to Ang II occurred via the AT_1 receptor.

The dose dependence of PLD activation in response to Ang II was then investigated. Again, [^{3}H]oleate-labelled primary glomerulosa cells were stimulated for 30 min in the presence of 0.5% ethanol and various concentrations of Ang II and the lipids were extracted and quantified. A slight but non-significant increase in radioactively labelled PEt levels was observed at a dose of 0.1 nM Ang II (Figure 1). PEt levels increased with increasing Ang II concentrations to attain a plateau of approx. two-fold over control at 10 nM Ang II and then fell slightly at 1 μM Ang II. This dose dependence mirrored the dose dependence of aldosterone secretion [17], suggesting that PLD activity is involved in mediating steroidogenesis in response to Ang II.

Table 1 Ang II activates PLD via the AT_1 receptor

[^{3}H]Oleate-prelabelled cells were incubated for 30 min in the presence or absence of 10 nM Ang II with or without 10 μM losartan or PD 123319, and in the presence of 0.5% (v/v) ethanol. Control cells were incubated with KRB$^+$ and 0.5% (v/v) ethanol. Reactions were terminated by the addition of 0.2% (w/v) SDS containing 5 mM EDTA, and [^{3}H]PA and PEt were extracted and were separated by TLC. Values are expressed as a ratio of control values and represent the means ($\pm$S.E.M.) of 6–9 samples from three separate experiments. Control values for PA were 17000$\pm$13000 c.p.m./mg of protein, and for PEt were 11000$\pm$9400 c.p.m./mg of protein. *$P < 0.01$ versus the control value.

Treatment	PA	PEt
Control	1.00$\pm$0.05	1.00$\pm$0.13
Ang II	1.73$\pm$0.18*	1.81$\pm$0.11*
Ang II + losartan	1.39$\pm$0.07	1.27$\pm$0.13
Ang II + PD 123319	1.65$\pm$0.14*	1.94$\pm$0.19*
Losartan	1.15$\pm$0.11	1.16$\pm$0.17
PD 123319	1.22$\pm$0.15	1.11$\pm$0.23

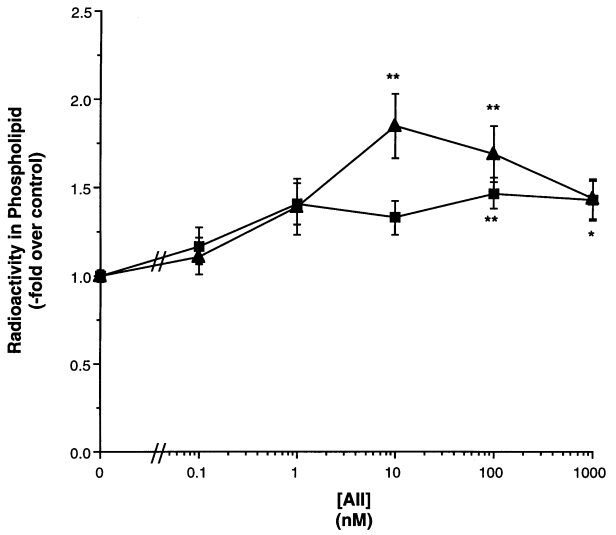

Figure 1 Ang II activates PLD in a dose-dependent manner

The [^{3}H]oleate-prelabelled cells were incubated for 30 min with the indicated concentrations of Ang II (AII) and 0.5% (v/v) ethanol. Control cells were incubated with KRB$^+$ and 0.5% (v/v) ethanol. Reactions were terminated by the addition of 0.2% (w/v) SDS containing 5 mM EDTA, and [^{3}H]PA (■) or PEt (▲) were extracted, separated by TLC and quantified as described in the Materials and methods section. Values are expressed as a ratio of the controls and represent the means ($\pm$S.E.M.) of 7–10 samples from four separate experiments; *$P < 0.05$, **$P < 0.01$ versus the control value. Control values for PA were 15000$\pm$6400 c.p.m./mg of protein and for PEt were 6100$\pm$1300 c.p.m./mg of protein.

In order for PLD to be involved in Ang II-elicited sustained aldosterone secretion, the enzyme must remain active throughout stimulation with the hormone. The time course of PLD activation in response to 100 nM Ang II is shown in Figure 2(A). The [^{3}H]oleate-prelabelled primary glomerulosa cells were exposed to Ang II for 0, 15 or 30 min before the addition of 0.5% (v/v) ethanol and incubation was continued for 30 min. Control cells were incubated in the absence of Ang II before 0.5% (v/v) ethanol was added to the medium. Additional time points in which cells were incubated with 0.5% (v/v) ethanol in the presence or absence of Ang II for 5 or 15 min were also performed. By this method, continued PEt production could be monitored over a 30-min time period, rather than determination of total PEt accumulated over 60 min. As shown, Ang II increased PEt formation after as little as 5 min of exposure to the hormone, and, in fact, the rate of PEt production over these first 5 min was greater than at later time points. A plateau enhancement of PEt levels of about two-fold over control was attained by about 30 min and was sustained throughout the 60-min period of stimulation. The time course of PLD activation in response to a lower concentration of Ang II (10 nM) was also determined. In these experiments, [^{3}H]oleate-prelabelled cells were preincubated for 0, 15 or 45 min in the presence or absence of Ang II before addition of 0.5% (v/v) ethanol for the final 15 min. As illustrated in Figure 2(B), PEt formation and PLD activation was sustained for the entire 60-min exposure with this lower Ang II dose.

In contrast, exposure of glomerulosa cells to 100 μM carbachol resulted in an initial increase in PEt levels which was maximal after 5 min of exposure and declined thereafter (Figure 3A). In fact, based on the observed absence of PEt production above the

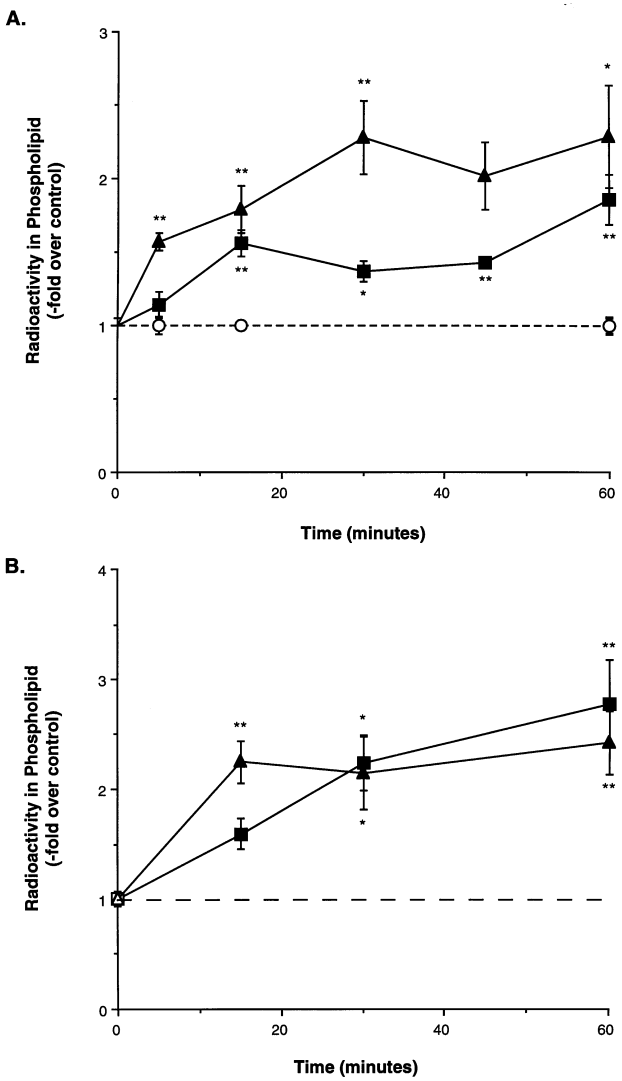

Figure 2 Ang II induced sustained PLD activation in bovine adrenal glomerulosa cells

(**A**) [^{3}H]Oleate-prelabelled cells were incubated for 0, 15 or 30 min with 100 nM Ang II before the addition of 0.5% (v/v) ethanol followed by a further incubation period of 30 min. Control cells (○) were incubated with KRB$^+$ and with 0.5% (v/v) ethanol for the final 30 min. Additional samples were incubated in the presence and absence of 100 nM Ang II and 0.5% (v/v) ethanol for 5 or 15 min. At the same time, 0.2% (w/v) SDS containing 5 mM EDTA was added to all samples and [^{3}H]PA (■) and PEt (▲) were extracted and separated by TLC. Values are expressed as a ratio of the appropriate control and represent the means (±S.E.M.) of 4–6 samples from two or three separate experiments; $^*P < 0.05$, $^{**}P < 0.01$ versus the appropriate control value. Control values for PA were 33000±15000 c.p.m./mg of protein and for PEt were 8200±1700 c.p.m./mg of protein at 5 min; 20000±8500 c.p.m./mg of protein (PA) and 5700±2000 c.p.m./mg of protein (PEt) at 15 min and 21000±9400 c.p.m./mg of protein (PA) and 6100±2800 c.p.m./mg of protein (PEt) for the 30–60 min time points. (**B**) [^{3}H]Oleate-prelabelled cells were incubated for 0, 15 or 45 min with 10 nM Ang II before the addition of 0.5% (v/v) ethanol followed by a further incubation period of 15 min. Control cells (time 0) were incubated with KRB$^+$ and with 0.5% (v/v) ethanol for the final 15 min. All reactions were terminated at the same time by the addition of 0.2% (w/v) SDS containing 5 mM EDTA. [^{3}H]PA (■) and PEt (▲) were extracted and separated by TLC. Values are expressed as a ratio of the appropriate control and represent the means (±S.E.M.) of 6–8 samples from three separate experiments; $^*P < 0.05$, $^{**}P < 0.001$ versus the appropriate control value. Control values for PA were 13000±9800 c.p.m./mg of protein and for PEt were 3400±2200 c.p.m./mg of protein.

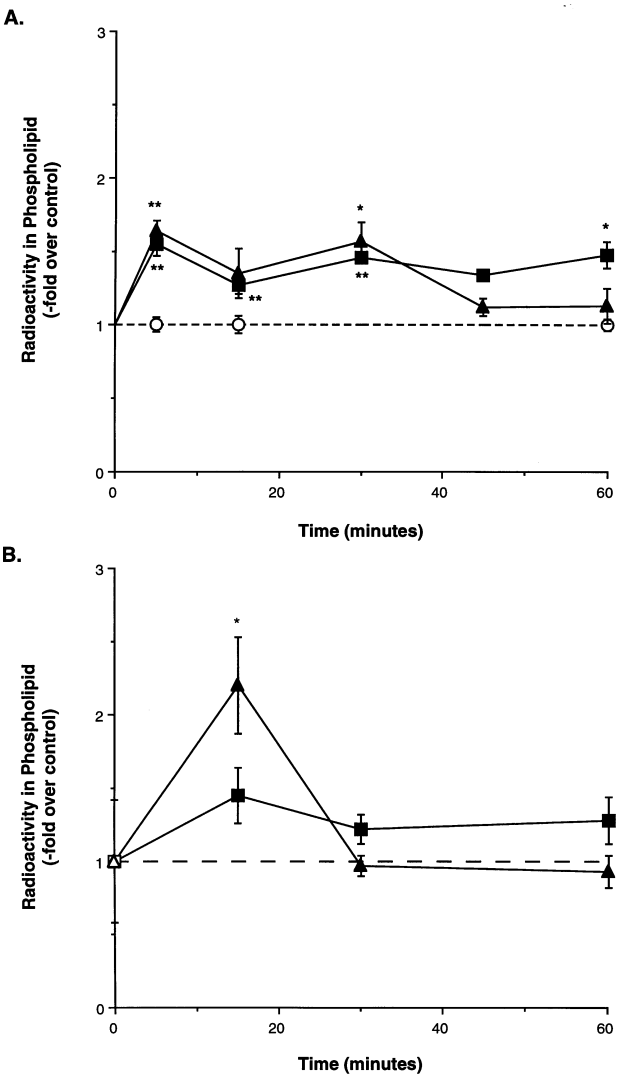

Figure 3 Carbachol activates PLD only transiently

(**A**) [^{3}H]Oleate-prelabelled cells were incubated for 0, 15 or 30 min with 100 μM carbachol before the addition of 0.5% (v/v) ethanol and a further incubation period of 30 min. Control cells (○) were incubated with KRB$^+$ and with 0.5% (v/v) ethanol for the final 30 min. Additional samples were incubated in the presence and absence of 100 μM carbachol and 0.5% (v/v) ethanol for 5 or 15 min. At the same time, 0.2% (w/v) SDS containing 5 mM EDTA was added to all samples and [^{3}H]PA (■) and PEt (▲) were extracted and were separated by TLC. Values are expressed as a ratio of the appropriate control and represent the means (±S.E.M.) of 4–6 samples from two or three separate experiments; $^*P < 0.05$, $^{**}P < 0.005$ versus the appropriate control value. Control values for PA were 17000±7000 c.p.m./mg of protein and for PEt were 6800±2000 c.p.m./mg of protein at 5 min, 20000±9600 c.p.m./mg of protein (PA) and 9700±4900 c.p.m./mg of protein (PEt) at 15 min and 12000±4800 c.p.m./mg of protein (PA) and 5300±2200 c.p.m./mg of protein (PEt) for the 30–60 min time points. (**B**) [^{3}H]Oleate-prelabelled cells were incubated for 0, 15 or 45 min with 1 mM carbachol before the addition of 0.5% (v/v) ethanol followed by a further 15 min incubation period. Control cells (time 0) were incubated with KRB and with 0.5% (v/v) ethanol for the final 15 min. All reactions were terminated at the same time by the addition of 0.2% (w/v) SDS containing 5 mM EDTA. [^{3}H]PA (■) and PEt (▲) were extracted and separated by TLC. Values are expressed as a ratio of the appropriate control and represent the means (±S.E.M.) of six samples from three separate experiments; $^*P < 0.01$ versus the control value. Control values for PA were 7900±3700 c.p.m./mg of protein and for PEt were 2700±1000 c.p.m./mg.

control level in cells pretreated for 15 min with carbachol before addition of ethanol for the final 30 min (45 min on Figure 3A), it can be argued that PLD is activated by carbachol only during the first ⩽15 min of exposure to this agonist. The transience of the PLD response to carbachol was not the result of a submaximal concentration of the agent, as 1 mM carbachol also generated a

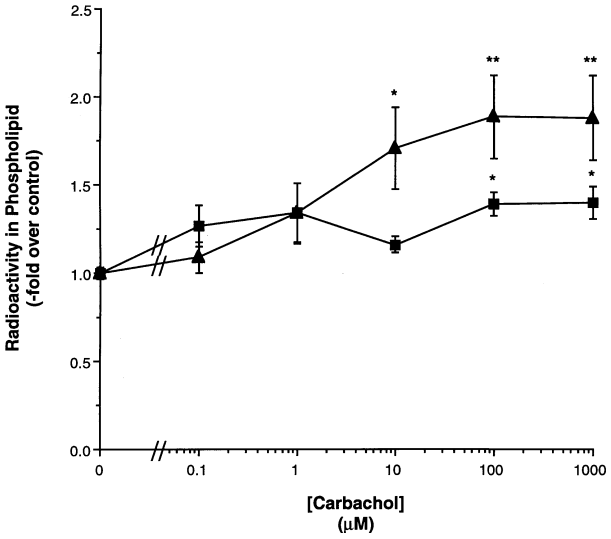

Figure 4 Carbachol activates PLD in a dose-dependent manner

[^{3}H]Oleate-prelabelled cells were incubated for 5 min with the indicated doses of carbachol and 0.5% (v/v) ethanol. Control cells were incubated with KRB$^+$ and 0.5% (v/v) ethanol. The reactions were terminated by the addition of 0.2% (w/v) SDS containing 5 mM EDTA, and [^{3}H]PA (■) and PEt (▲) were extracted and separated by TLC. Values are expressed as a ratio of controls and represent the means (±S.E.M.) of six samples from three separate experiments; $^*P < 0.05$, $^{**}P < 0.01$ versus the control value. Control values for PA were 19900±1500 c.p.m./mg of protein and for PEt were 5700±450 c.p.m./mg of protein.

PLD activation, which was complete within the first 15 min of exposure (Figure 3B). Furthermore, in dose-response experiments, 100 μM carbachol elicited maximal PEt formation after 5 min of exposure (Figure 4). Thus our results suggest that the activation of PLD by Ang II is sustained and that by carbachol is transient.

DISCUSSION

The ability of losartan to inhibit the Ang II-elicited activation of PLD (Table 1) is not unexpected. The AT_1 receptor inhibited by this compound is known to mediate aldosterone secretion as well as the mitogenic effects of Ang II [20,21]. On the other hand, the physiological role of the AT_2 receptor is unclear [20]. Studies with knockout mice, however, suggest that Ang II binding to the AT_2 receptor triggers a feedback inhibition that counters the effects of the AT_1 receptor to aid in blood pressure regulation, as well as central nervous system effects [22,23]. The signalling pathway used by this receptor subtype is unknown in human and bovine glomerulosa cells (although in rats guanylate cyclase is apparently inhibited by the AT_2 receptor) [20] but apparently does not involve PLD activation, since we observed no significant effect of the AT_2 inhibitor, PD 123319, on PEt production. That activation of PLD occurs via the AT_1 receptor supports our hypothesis that the activity of this enzyme plays a role in mediating sustained Ang II-induced aldosterone secretion.

Likewise, our finding that the dose dependence of Ang II-induced PEt formation (Figure 1) paralleled the dose response for Ang II-mediated aldosterone secretion [19] suggests a possible role for PLD activity in regulating the aldosterone secretory response. Further evidence for this idea is provided by the ability of Ang II to elicit sustained PLD activation (Figure 2). Thus maintained PLD activation should result in sustained generation of PA which can then be dephosphorylated by PA phosphohydrolase to yield DAG, a signalling molecule thought to be critically important in the regulation of steroidogenesis [24]. In other systems it has been possible to inhibit PA phosphohydrolase using the compound propranolol (in the concentration range 10–100 μM), with consequent inhibition of DAG levels [12,25,26]. We attempted to use propranolol to inhibit glomerulosa PA phosphohydrolase and found, indeed, that this compound (100 μM) reduced the Ang II-elicited increase in DAG content by approximately 25% ($n = 5$; $P < 0.05$; E. M. Jung and W. B. Bollag, unpublished work). Unfortunately, we found that this compound inhibited Ang II-induced and adrenocorticotropic hormone (ACTH)-elicited steroidogenesis (E. M. Jung and W. B. Bollag, unpublished work). Since ACTH elicits aldosterone secretion via the cAMP pathway [3] and does not activate PLD (W. B. Bollag, unpublished work), this result suggests a potential non-specific cytotoxic effect of propranolol. Alternatively, while ACTH may not itself activate PLD to increase DAG levels indirectly through PA phosphohydrolase, a minimal basal DAG generation by the combined PLD and PA phosphohydrolase activities may be essential for the action of this hormone. In subsequent experiments we attempted to prevent DAG formation via PLD by diverting production from PA to PEt in the presence of ethanol. After a 15-min incubation, ethanol (1%, v/v) alone appeared to increase DAG content slightly (33%, $n = 3$; $P < 0.05$), perhaps due to an activation of PLD, as has been observed previously in mesangial cells [26], and had no effect on the Ang II-induced increase in DAG content (E. M. Jung and W. B. Bollag, unpublished work). Furthermore, we found that, like propranolol, ethanol also appeared to exhibit non-specific cytotoxicity; concentrations of 0.5% and 1% (v/v) [25] significantly inhibited Ang II-induced aldosterone secretion by 23 and 35% respectively ($n = 5$; $P < 0.01$ for both values). However, these concentrations of ethanol also slightly (but not significantly) inhibited ACTH-elicited secretion (by 12 and 21%, $n = 5$; $P > 0.05$ for both values) and significantly inhibited 10 mM potassium-stimulated steroidogenesis (by 24 and 54%, $n = 3$; $P < 0.05$ for both values; E. M. Jung and W. B. Bollag, unpublished work).

PLD exhibits activation characteristics that are consistent with a role for this enzyme in sustained DAG production and aldosterone secretion. There are also data in the literature to support this idea. Hunyady et al. [1] have demonstrated previously that removal of extracellular calcium from the medium completely inhibited inositol phosphate production but only partially inhibited DAG levels (by approx. one third). These data suggest that up to two thirds of the sustained increase in DAG content may be provided by a mechanism that does not involve phosphoinositide hydrolysis, and we propose that this phosphoinositide-independent DAG generation occurs via the actions of PLD and PA phosphohydrolase. (However, the possible involvement of a PC-PLC has not been investigated to date.) Consistent with this hypothesis, we have observed, following removal of Ang II, that there is a persistent elevation in DAG representing approx. two thirds of the Ang II-elicited increase [17]. We demonstrated that this persistent enhancement is the result of a species of DAG, marked by myristate, that has a slow turnover, whereas a DAG pool for which arachidonate serves as a marker is turned over rapidly [17]. The source of this myristate-containing DAG is likely to be phosphatidylcholine, the phospholipid into which the majority of the myristate radiolabel is incorporated [17]. Since phosphatidylcholine is often a substrate of PLD [13–16], the maintained activity of PLD in response to Ang II (Figure 2) may explain not only the hormone-induced production of myristate-containing DAG but also our previous

observation that Ang II elicits sustained phosphatidylcholine hydrolysis [17].

We have proposed also that the persistent increase in DAG, the result of the production of slowly turned over myristate-containing DAG, is responsible for the ability of Ang II to induce priming. Priming is the process by which pretreatment with and removal of Ang II sensitizes glomerulosa cells to respond to a second agonist with enhanced aldosterone secretion. Thus a second Ang II stimulus in Ang II-pretreated glomerulosa cells results in a secretory rate of approximately 150% of that seen in non-pretreated cells [24]. Similarly, the Ca^{2+} channel agonist, Bay K8644, triggers aldosterone secretion in Ang II-pre-exposed glomerulosa cells, whereas the response of naive, unpretreated cells is minimal [17,24]. It was suggested that priming was the result of a maintained association of PKC at the plasma membrane. Hence, following Ang II removal, PKC would remain at the membrane where it could be activated by an increase in Ca^{2+} influx initiated by Bay K8644 or a second addition of Ang II, as shown by Kojima et al. [6]. Since the association of PKC with the plasma membrane is promoted physiologically by DAG, we proposed that the persistent elevation in DAG following Ang II removal mediated the induction of priming [17]. Based on the data reported herein we further propose that the persistent, i.e. slowly turned over DAG, which underlies priming is derived from the combined action of PLD and PA phosphohydrolase.

The inability of carbachol to elicit sustained PLD activation (Figure 3) would suggest that the removal of this agent should not be accompanied by a persistent elevation in DAG content. In fact, our previous data indicate carbachol is less effective than Ang II in generating myristate-containing DAG and that the carbachol-induced change in DAG is not maintained following hormone removal. Furthermore, carbachol is unable to induce priming [9]. In other systems PLD exhibits agonist-specific attentuation mechanisms [27,28], however, these studies showed transient activation of PLD by physiological agonists and sustained activation by phorbol esters. Our results provide evidence for alternate mechanisms of activation and/or inactivation for two physiologically relevant agonists and suggest that these differences in coupling may lead to functional variability in a cellular response. Furthermore, the data suggest that the inability of carbachol to sustain PLD activation (Figure 3) is responsible for the lack of induction of priming by this agent.

Differences have been described in the ability of DAG derived from PLD/PA phosphohydrolase action versus PI-PLC to mediate specific cellular responses. Thus in pituitary gonadotrophs transcriptional activation of c-*fos* expression appears to be coupled to DAG produced via PLD/PA phosphohydrolase but not PI-PLC, whereas luteinizing hormone secretion requires DAG derived from PI-PLC activity rather than PLD/PA phosphohydrolase [25]. This difference may be the result of differential activation of various PKC isoenzymes by the two DAG species produced as the consequence of the activity of PLD or PI-PLC on their respective substrates. Alternatively, the two DAG pools may be differentially metabolized so that their levels vary temporally to affect cell processes. Alternatively, the two DAG species may be generated in different cellular locations to influence hormone-induced responses according to their spatial pattern. Indeed, Balboa and Insel have described an ATP-activated PLD localized to the nuclei of MDCK-D1 cells [29]. Thus DAG generated in the nucleus by PLD/PA phosphohydrolase might alter nuclear processes such as transcription, as observed in the pituitary gonadotrophs [25], presumably through recruitment of PKC to these sites. Localization of DAG production and PKC recruitment to other specific cell regions with specialized enzymic machinery might also permit certain responses to be mediated by PLD/PA phosphohydrolase and others by PI-PLC.

Our data do not prove a role for PLD in steroidogenesis or priming. Indeed, it is possible that Ang II-induced DAG generation via PLD/PA phosphohydrolase serves an alternate function, such as promoting transcription of proteins (e.g. the steroidogenic acute regulatory protein [30]) necessary for steroidogenesis or trophic maintenance or growth [31] of the glomerulosa cell. Definitive evidence for involvement of PLD in steroidogenesis and/or growth regulation awaits the discovery of a specific (and non-toxic) inhibitor and/or molecular biological approaches to reduce its function. Nevertheless, our results are entirely consistent with the hypothesis that the activity of this enzyme mediates the Ang II-elicited sustained aldosterone secretory response as well as the persistent increase in myristate-containing DAG and induction of priming by this hormone. Further studies are necessary to determine whether myristate-containing DAG remains elevated because its production is maintained or its degradation is delayed relative to arachidonate-enriched DAG. Future experiments will address this issue and the ability of myristate-containing DAG to activate PKC and maintain its association with the plasma membrane.

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