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Rate of calcium entry determines the rapid changes in protein kinase C activity in angiotensin II-stimulated adrenal glomerulosa cells

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The present study was conducted to monitor precisely the activity of protein kinase C (PKC) in adrenal glomerulosa cells stimulated by angiotensin II (ANG II). PKC activity in cells was monitored by measuring phosphorylation of a synthetic KRTLRR peptide, a specific substrate for PKC, immediately after the permeabilization of the cells with digitonin [Heasley and Johnson J. Biol. Chem. (1989) **264**, 8646–8652]. Addition of 1 nM ANG II induced a gradual increase in KRTLRR peptide phosphorylation, which reached a peak at 30 min, and phosphorylation was sustained thereafter. When the action of ANG II was terminated by adding [Sar^1,Ala^8]ANG II, a competitive antagonist, both Ca^{2+} entry and KRTLRR phosphorylation ceased rapidly, whereas diacylglyercol (DAG) content was not changed significantly within 10 min. Similarly, when blockade of Ca^{2+} entry was achieved by decreasing extracellular Ca^{2+} to 1 μM or by adding 1 μM nitrendipine, KRTLRR peptide phosphorylation was decreased within 5 min. In addition, restoration of Ca^{2+} entry was accompanied by an immediate increase in KRTLRR peptide phosphorylation. Under the same condition, DAG content did not change significantly. We then examined the role of the PKC pathway in ANG II-induced aldosterone production. Ro 31-8220 inhibited ANG II-induced KRTLRR phosphorylation without affecting the activity of calmodulin-dependent protein kinase II. In the presence of Ro 31-8220, ANG II-mediated aldosterone production was decreased to approx. 50 %. Likewise, intracellular administration of PKC_{19-36}, a sequence corresponding to residues 19–36 of the regulatory domain of PKC known to inhibit PKC activity, attenuated ANG II-mediated activation of PKC and aldosterone output. These results indicate a critical role of Ca^{2+} entry in the regulation of PKC activity by ANG II.

INTRODUCTION

Protein-kinase C (PKC) is a family of Ca^{2+}-activated phospholipid-dependent protein kinases and plays a pivotal role in the signal-transduction pathways activated by variety of agonists (see [1] for review). On stimulation of cells with a Ca^{2+}-mobilizing agonist, PtdIns(4,5)P_2 is cleaved by the enzyme phospholipase C, which leads to the generation of Ins(1,4,5)P_3 and 1,2-diacylglycerol (DAG) [2]. DAG activates PKC by increasing the sensitivity of the enzyme to Ca^{2+}. Additionally, a bolus of Ca^{2+} released from an intracellular pool by Ins(1,4,5)P_3 supports the activation of PKC, presumably through a mechanism involving translocation of PKC to the plasma membrane [3]. Ca^{2+} entry induced by the agonist may increase the concentration of Ca^{2+} in the sub-plasma-membrane domain and is thought to be important for the maintenance of the activity of PKC. Although breakdown of phosphoinositides is critical for the initiation, DAG is subsequently supplied by agonist-induced breakdown of phosphatidylcholine, and the DAG thus provided may be also important for the activation of PKC. The activity of PKC is also thought to be supported by fatty acids released via an activation of phospholipase A_2 [4]. The sequence of events after addition of the Ca^{2+}-mobilizing agonist is well characterized, but only limited information is currently available about the changes in the activity of PKC after stimulation of the cells with a Ca^{2+}-mobilizing agonist [5–7]. This is largely due to technical difficulty in monitoring the activity of PKC precisely in activated cells.

In the present study, we measured the changes in PKC activity in adrenal glomerulosa cells after the stimulation with a Ca^{2+}-mobilizing agonist, angiotensin II (ANG II). The results indicate that PKC is activated persistently in ANG II-stimulated cells. Ca^{2+} entry induced by ANG II plays a critical role in the maintenance of the activity of PKC by the agonist.

EXPERIMENTAL

Cell culture

Bovine adrenal glomerulosa cells were prepared by using collagenase [8] and were cultured in Dulbecco's modified Eagle's medium containing 10 % calf serum (Gibco, Grand Island, NY, U.S.A.), 1 μM ascorbate, 1 μM α-tocopherol, 1 μM metyrapone, 100 m-units/ml penicillin and 100 mg/ml streptomycin [9]. For stimulation with ANG II, cells were incubated in Krebs–Ringer bicarbonate buffer containing 1.25 mM Ca^{2+}, 3.5 mM K^+, 5.5 mM glucose and 1 % BSA (KRB buffer) equilibrated with O_2/CO_2 (19:1).

Measurement of aldosterone output

Cells cultured in a 24-well plate overnight were preincubated in KRB buffer for 30 min. Cells were then incubated for the indicated time in KRB buffer with various agents and the supernatant was collected. For time-course experiments, supernatant was removed every 15 min and replaced with KRB buffer containing the same agent. Aldosterone was measured by radioimmunoassay [8].

Measurement of activity of PKC

Activity of PKC was monitored with synthetic KRTLRR peptide as a substrate, by the method by Heasley and Johnson [5] as

Abbreviations used: PKC, protein kinase C; ANG II, angiotensin II; DAG, 1,2-diacylglycerol; KRB buffer, Krebs–Ringer bicarbonate buffer; PKC_{19-36}, a synthetic polypeptide corresponding to residues 19–36 of the regulatory domain of PKC; PMA, 4-phorbol 12-myristate 13-acetate; diC_8, dioctanoylglycerol; kinase II, calmodulin-dependent protein kinase II.

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described previously [10]. In short, cells cultured in a 24-well plate were washed with KRB buffer. The cells were incubated at 37 °C for the indicated time in the presence of various agents. The solution was then aspirated and replaced with 200 μl of salt solution (137 mM NaCl, 5.4 mM KCl, 0.3 mM sodium phosphate, 0.4 mM potassium phosphate, 5.5 mM glucose and 20 mM Hepes) supplemented with 50 μg/ml digitonin, 10 mM $MgCl_2$, 25 mM β-glycerophosphate, 100 μM [γ-^{32}P]ATP and 300 μM KRTLRR peptide. The Ca^{2+} concentration in the solution was adjusted to 100 nM with Ca^{2+}–EGTA buffer [10] unless otherwise mentioned. The reaction was terminated after a 10 min incubation at 30 °C by adding 50 μl of 25% trichloroacetic acid. Samples of acidified reaction mixtures were spotted on 2 cm × 2 cm phosphocellulose squares (Whatman P-81) and washed three times with 75 mM H_3PO_4 and once with 75 mM sodium phosphate (pH 7.5). Peptides were synthesized with an automated solid-phase peptide synthesizer and were verified by gas-phase sequencing and amino acid analysis.

Measurement of calmodulin-dependent protein kinase II activity (kinase II)

The protein kinase activity was assayed by measuring ^{32}P transfer from [^{32}P]ATP to synapsin I. The assay mixture (0.1 ml) contained 20 mM Tris/malate (pH 6.8), 10 mM $MgCl_2$, 3 mM EDTA, substrate protein and enzyme. The concentrations of free Ca^{2+} and calmodulin were 200 μM and 10 μg/ml respectively. The reaction was initiated by adding [^{32}P]ATP (20 μM, 2.5 Ci/mmol), carried out for various lengths of time at 37 °C and terminated by adding 50 μl of stop solution containing 30 mM EDTA and 5% SDS. Samples were boiled at 100 °C for 5 min, and 10 μl of 1 M dithiothreitol was added before electrophoresis. Phosphorylated proteins were analysed by SDS/PAGE. For quantification, proteins were identified by Coomassie Blue staining and excised from dried gels, and were counted for radioactivity by liquid-scintillation spectrometry. Calmodulin-dependent kinase II was isolated from cytosol of the glomerulosa layer as described by Gorelick et al. [11].

Measurement of DAG

For measurement of DAG mass, cells were grown in 60 mm dishes and preincubated for 30 min in KRB buffer. Cells were incubated for the indicated time with ANG II and were then scraped off by using a rubber policeman. Lipids were extracted by adding chloroform/methanol (1:2, v/v) and DAG mass was measured as described by Preiss et al. [12].

Loading of PKC_{19-36} into cells

A synthetic polypeptide corresponding to residues 19–36 of regulatory domain of PKC (PKC_{19-36}), which specifically blocks the activity of PKC [13], was loaded into freshly isolated glomerulosa cells by reversible permeabilization of the plasma membrane with ATP [14]. Cells were incubated in the following solutions sequentially for 20 min at 4 °C: solution I, 120 mM KCl/10 mM EGTA, 5 mM Na_2ATP/2 mM $MgCl_2$/20 mM Hepes (pH 7.1); solution II, 120 mM KCl/0.1 mM EGTA/5 mM Na_2ATP/2 mM $MgCl_2$/20 mM Hepes (pH 7.1)/50 μM PKC_{19-36} or PKC_{1-18}, a control peptide. Cells were then incubated for 40 min at 4 °C in a solution containing 120 mM KCl, 0.1 mM EGTA, 5 mM Na_2ATP, 10 mM $MgCl_2$ and 20 mM Hepes (pH 7.1). Cells were centrifuged and were then incubated for 60 min at room temperature in a solution containing 120 mM NaCl, 3.5 mM KCl, 5 mM $NaHCO_3$, 1.4 mM NaH_2PO_4, 10 mM $MgCl_2$, 10 mM Hepes (pH 7.4) and 5.5 mM glucose. PKC_{19-36}-loaded cells were then cultured overnight. PKC activity and aldosterone output were then measured in PKC_{19-36}-loaded cells.

RESULTS

Effect of DAG and calmodulin on KRTLRR peptide phosphorylation

We employed KRTLRR peptide, a sequence corresponding to a PKC phosphorylation site in epidermal-growth-factor receptor, as a substrate to measure the activity of PKC [5]. As demonstrated in Table 1, KRTLRR peptide phosphorylation was enhanced by an addition of dioctanoylglycerol (diC_8), but not by a combination of Ca^{2+} and calmodulin. Also, KRTLRR peptide phosphorylation was blocked by PKC_{19-36}, a peptide which specifically blocks the activity of PKC [13]. Therefore, as in previous reports [5,10], KRTLRR peptide phosphorylation was caused largely by PKC.

Effect of phorbol ester and DAG on KRTLRR phosphorylation

Figure 1 demonstrates the time course of the effect of phorbol ester and DAG on KRTLRR peptide phosphorylation. Cells were incubated with 10 nM 4-phorbol 12-myristate 13-acetate (PMA) for the indicated time, and KRTLRR phosphorylation was then measured after permeabilization with digitonin. As

Table 1 Effect of DAG and calmodulin on KRTLRR phosphorylation

Unstimulated glomerulosa cells were lysed by adding digitonin, [^{32}P]ATP and KRTLRR. The phosphorylation was measured by adding either 25 μM diC_8 or 1 nM calmodulin. Ca^{2+} concentration in the reaction mixture was adjusted by using Ca–EGTA buffer. Values are means ± S.E.M. for three determinations

Addition	KRTLRR peptide phosphorylation (pmol/min per mg of protein)
None	1.2 ± 0.4
300 nM Ca^{2+}	1.5 ± 0.5
300 nM Ca^{2+} + diC_8	7.6 ± 1.2
1 μM Ca^{2+}	1.5 ± 0.3
1 μM Ca^{2+} + calmodulin	1.4 ± 0.6

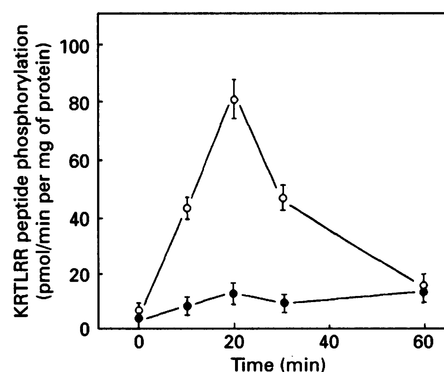

Figure 1 Time course of effect of phorbol ester and DAG on KRTLRR peptide phosphorylation

Cells were incubated for the indicated time with 10 nM PMA (○) or 25 μM diC_8 (●). Cells were then lysed by adding digitonin, [^{32}P]ATP and KRTLRR peptide. Phosphorylation of the peptide was measured. Values are means ± S.E.M. for four experiments.

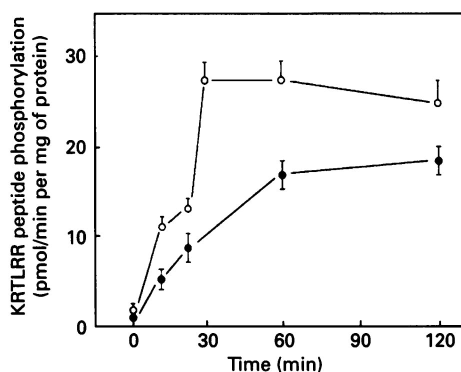

Figure 2 Time course of effect of ANG II on KRTLRR peptide phosphorylation

Cells were incubated for the indicated time with 1 nM (○) or 100 pM (●) ANG II. Cells were then lysed and KRTLRR peptide phosphorylation was measured. Values are means ± S.E.M. for three experiments.

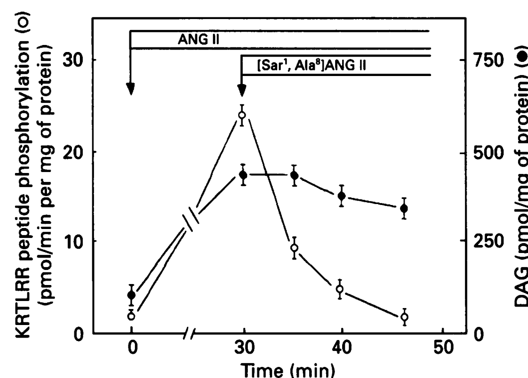

Figure 4 Time course of changes in KRTLRR peptide phosphorylation and DAG content after termination of ANG II action

Cells were incubated for 30 min with 1 nM ANG II, and the action was blocked by adding 100 nM [Sar^1,Ala^8]ANG II at 30 min. KRTLRR phosphorylation and DAG content were measured. Values are means ± S.E.M. for three experiments.

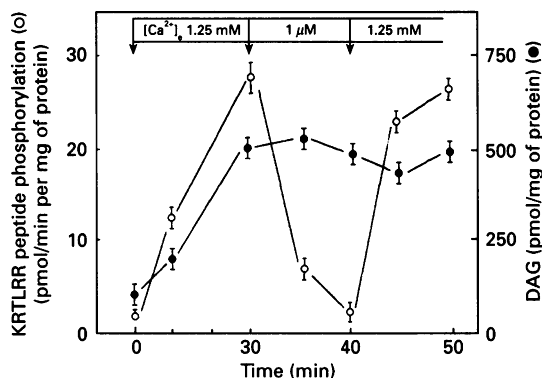

Figure 3 Effect of decrease in extracellular Ca^{2+} on ANG II-induced KRTLRR peptide phosphorylation and DAG content

Cells were incubated for the indicated time with 1 nM ANG II. Extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) was decreased to 1 μM from 30 to 40 min. Cells were lysed at each time point, and KRTLRR peptide phosphorylation (○) and DAG content (●) were measured. Values are means ± S.E.M. for three experiments.

depicted, addition of PMA resulted in a marked increase in KRTLRR phosphorylation. The effect of PMA was maximal at 20 min, and declined thereafter. In contrast with the effect of PMA, the time course of actions of a synthetic DAG was different; diC_8 induced a small elevation of KRTLRR phosphorylation, which persisted for 60 min.

Time course of changes in KRTLRR peptide phosphorylation in ANG II-stimulated glomerulosa cells

To monitor the time course of the activation of PKC in ANG II-stimulated cells, we monitored changes in KRTLRR peptide phosphorylation. Cells incubated for various times with 1 nM ANG II were lysed by adding digotinin. KRTLRR peptide and [^{32}P]ATP were added together with digitonin, and ^{32}P incorporated into KRTLRR peptide was measured. As shown in Figure 2, KRTLRR phosphorylation was increased by 1 nM ANG II. An increase in KRTLRR peptide phosphorylation was evident at 10 min, and the phosphorylation increased rather slowly. At 30 min after addition of ANG II, KRTLRR phosphorylation reached the highest value and remained there for at least 120 min. A similar pattern of changes was observed in cells stimulated by 100 pM ANG II.

Effect of blockade of Ca^{2+} entry on ANG II-induced KRTLRR phosphorylation

Our previous observations suggest that ANG II-induced activation of PKC depends greatly on Ca^{2+} entry [15–18]. To determine the dependence of agonist-induced activation of PKC on Ca^{2+} entry, we measured the changes in PKC activity after the blockade of ANG II-induced Ca^{2+} entry. Cells were incubated for 30 min with 1 nM ANG II in KRB buffer containing 1.25 mM Ca^{2+}. At 30 min, the incubation medium was changed to KRB buffer containing 1 μM Ca^{2+} and 1 nM ANG II. A previous study indicates that ANG II does not stimulate Ca^{2+} entry at this concentration of extracellular Ca^{2+} [15]. As shown in Figure 3, termination of Ca^{2+} entry caused a rapid decrease in KRTLRR peptide phosphorylation within 5 min. Similar results were obtained when Ca^{2+} entry was blocked by addition of 1 μM nitrendipine (results not shown). When extracellular Ca^{2+} was restored 10 min after the decrease in extracellular Ca^{2+}, KRTLRR peptide phosphorylation recovered very rapidly: it reached near the peak value within 5 min, and the time course of this was quite different from that at the beginning of stimulation by ANG II.

To ascertain that the decrease in KRTLRR phosphorylation after the blockade of Ca^{2+} entry was not due to a decrease in DAG, we measured the change in DAG mass after the termination of Ca^{2+} entry. As shown in Figure 3, ANG II elicited a 4-fold increase in DAG mass at 30 min. After the termination of Ca^{2+} entry, the DAG content remained elevated.

Time course of changes in KRTLRR peptide phosphorylation after termination of ANG II action

Previous studies indicate that Ca^{2+} entry ceases immediately by the blockade of ANG II action and aldosterone output falls rapidly after termination of ANG II action [15,16]. We then measured changes in PKC activity after the termination of ANG II action. Cells were incubated for 30 min with 1 nM ANG II, and then [Sar^1,Ala^8]ANG II, a competitive antagonist, was

Table 2 Effect of Ro 31-8220 on KRTLRR peptide phosphorylation

Cells were incubated for 120 min with 1 nM ANG II in the absence and presence of 100 nM Ro 31-8220. The medium was stored at −20 °C for measurement of aldosterone. Cells were lysed and KRTLRR phosphorylation was determined. Values are means ± S.E.M. for three experiments.

Addition	KRTLRR phosphorylation (pmol/min per mg of protein)	Aldosterone output (ng/10^5 cells)
None	1.2 ± 0.4	4.3 ± 1.2
ANG II	14.3 ± 2.4	48.4 ± 5.4
ANG II + Ro 31-8220	2.5 ± 1.6	26.9 ± 7.2

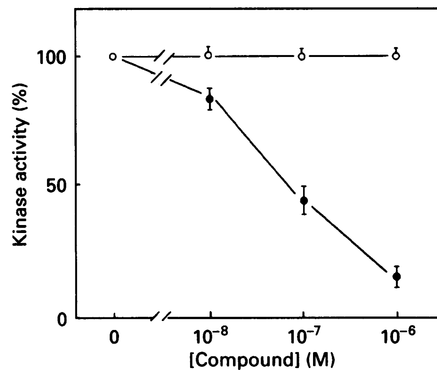

Figure 5 Effect of Ro 31-8220 and W-7 on the activity of kinase II

The activity of purified kinase II was measured in the presence of various concentrations of either Ro 31-8220 (○) or W-7 (●). Values are means ± S.E.M. for four determinations.

added to terminate the action of ANG II. As depicted in Figure 4, KRTLRR peptide phosphorylation was rapidly decreased after the termination of the action of ANG II, and returned to the basal value within 10 min. We then measured changes in DAG mass by the same protocol. As shown in Figure 4, DAG mass was decreased only slightly at 10 min after the termination of ANG II action.

Effect of blockade of PKC action on ANG II-induced aldosterone secretion

We proposed that activation of PKC is essential for the sustained production of aldosterone in ANG II-treated cells [19]. However, controversy still exists as to the role of PKC in ANG II-induced aldosterone production [20,21]. Using the sensitive and accurate assay system for PKC activity in intact cells, we re-evaluated the role of PKC in the action of ANG II.

First, we employed Ro 31-8220, a relatively specific inhibitor of PKC [22], which effectively blocks the activity of PKC in intact cells [10,22,23]. As shown in Table 2, 100 nM Ro 31-8220 completely blocked ANG II-induced phosphorylation of KRTLRR peptide. In our culture system, ANG II induced a more than 10-fold increase in aldosterone production during 120 min. When 100 mM Ro 31-8220 was added together with ANG II, aldosterone output was markedly decreased. In the absence of PKC activation, as assessed by KRTLRR phosphorylation, aldosterone output induced by ANG II was decreased to approx. 50%. To determine that the inhibitory action of Ro 31-8220 is not due to its effect on the Ca^{2+}–calmodulin system, we performed two sets of experiments.

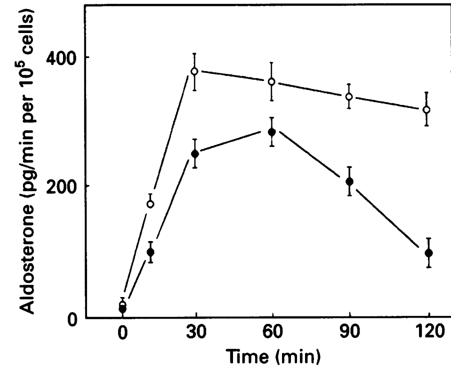

Figure 6 Effect of Ro 31-8220 in ANG II-mediated aldosterone production

Cells were incubated with 1 nM ANG II in the absence (○) and presence (●) of 100 nM Ro 31-8220 and time course of aldosterone output was measured as described in the Experimental section. Values are means ± S.E.M. for three experiments.

Table 3 Effect of ANG II in PKC_{19-36}-loaded cells

Cells were loaded with either PKC_{19-36} or a control peptide (Control cells) and were cultured overnight. Cells were then incubated with either 1 nM ANG II or 8 mM K^+, and KRTLRR phosphorylation and aldosterone output were measured. PMA (10 nM) was added in some experiments. Values are means ± S.E.M. for three experiments.

Addition	KRTLRR phosphorylation (pmol/min per mg of protein)	Aldosterone output (ng/10^5 cells)
Control cells		
None	2.0 ± 0.6	3.9 ± 1.2
ANG II	13.1 ± 2.4	42.4 ± 6.2
K^+	2.4 ± 0.9	24.3 ± 5.1
PKC_{19-36}-loaded cells		
None	1.6 ± 0.3	5.0 ± 1.6
ANG II	2.1 ± 0.7	29.4 ± 6.6
K^+	1.8 ± 0.2	26.3 ± 3.6
PKC_{19-36}-loaded cells		
None	2.2 ± 0.5	3.4 ± 1.4
ANG II	1.9 ± 0.4	31.6 ± 5.9
ANG II + PMA	2.0 ± 0.7	27.9 ± 8.2

Calmodulin-dependent protein kinase II (kinase II) is a major calmodulin-dependent enzyme in glomerulosa cells. Figure 5 shows the effect of Ro 31-8220 on the activity of kinase II assayed *in vitro*. As depicted, up to 1 μM Ro 31-8220 had no effect on kinase II activity. In contrast, W-7, an inhibitor of calmodulin [24], effectively blocked the activity of kinase II. In addition, when added into intact cells, Ro 31-8220 did not affect aldosterone output stimulated by a Ca^{2+} ionophore, A23187 (results not shown). Figure 6 demonstrates the time course of ANG II-induced aldosterone production in the absence and presence of 100 nM Ro 31-8220. In the presence of Ro 31-8220, the sustained phase of aldosterone output was greatly diminished. Moreover, the initial phase of aldosterone output was also decreased by Ro 31-8220.

Second, we employed the synthetic peptide PKC_{19-36}, a specific inhibitor of PKC, to block ANG II-induced activation of PKC. In PKC_{19-36}-loaded cells, ANG II did not stimulate KRTLRR phosphorylation, whereas administration of a control peptide did not affect ANG II-induced KRTLRR phosphorylation (Table 3). In these cells, ANG II induced a 6-fold increase in aldosterone output, whereas in control cells ANG II elicited

more than 10-fold stimulation. PKC_{19-36} did not affect aldosterone production induced by 8 mM K^+. In PKC_{19-36}-loaded cells, addition of PMA did not affect either ANG II-mediated KRTLRR phosphorylation or aldosterone output.

DISCUSSION

The present results provide new insights into the regulation of PKC in cells stimulated by a Ca^{2+}-mobilizing agonist. To date, only a limited number of studies have been reported on the time course of the activation of PKC in activated cells [5–7,10], and little is known about the precise changes in PKC activity after the stimulation. This is probably due to technical difficulties in monitoring the activity of PKC in intact cells for a long period. In the present study, we attempted to monitor the activity of PKC in intact adrenal glomerulosa cells by measuring phosphorylation of a synthetic substrate for PKC [5]. As has been shown previously [5,10], the activity measured with KRTLRR peptide as a substrate reflects mainly the activity of PKC in intact cells, although the type of isoenzyme of activated PKC is not identified by this method. As demonstrated in Table 1, KRTLRR phosphorylation is augmented by DAG, but not by calmodulin. Also, DAG-mediated stimulation is blocked by PKC_{19-36}, a specific inhibitor of PKC. Therefore it is reasonable that activity measured by using KRTLRR as a substrate represents mostly the activity of PKC in the cells. As shown in Figure 2, KRTLRR peptide phosphorylation increases rather slowly after the stimulation with ANG II. The results are in agreement with our previous observation that phosphorylation of PKC substrate in ANG II-stimulated cells does not occur at 1 min after stimulation, but becomes significant at 30 min [6]. The present results extend previous work by showing that ANG II induces persistent activation of PKC in glomerulosa cells. For the maintenance of PKC activity, ANG II-mediated Ca^{2+} entry plays an essential role. When the action of ANG II is terminated, both Ca^{2+} entry [15] and PKC activity fall quickly, whereas DAG content does not change significantly within 10 min. Also, when Ca^{2+} entry is blocked, facilitated PKC activity declines quickly even though cellular DAG content remains elevated. Hence the rate of Ca^{2+} entry determines the minute-to-minute regulation of PKC activity in stimulated cells. When Ca^{2+} entry is restored, PKC activity returns rapidly to the peak value. PKC activity is decreased by the blockade of Ca^{2+} entry, but PKC remains in a state where, in the presence of Ca^{2+} entry, it can be activated immediately. The molecular basis for such a state is not known; however, it is worth mentioning that activated PKC translocates to the plasma membrane [3,4]. Given that DAG remains elevated even when Ca^{2+} entry is blocked, it is possible that PKC remains in the plasma membrane when Ca^{2+} entry is blocked.

In the present study, we re-evaluated the role of PKC in ANG II-induced aldosterone production in adrenal glomerulosa cells. Our results indicate that, when PKC is effectively blocked by either Ro 31-8220 or PKC_{19-36}, ANG II-induced aldosterone output is considerably decreased. Furthermore, blockage of PKC activation attenuated not only the sustained phase but also the initial phase of aldosterone release. These results indicate the importance of the PKC pathway in ANG II-induced steroid production. Our results are different from those obtained by others [20,21], that PKC does not play a major role in ANG II-induced steroid production. The reasons for the discrepancy are not totally clear, but the following two points should be mentioned. First, in none of these studies is it determined, in a strict sense, whether PKC is in fact blocked by the procedures, either addition of inhibitors or down-regulation of PKC. In addition, down-regulation of PKC by pretreatment with phorbol ester sometimes leads to an incorrect conclusion [10]. As pointed out previously [9], pretreatment with phorbol ester alters phospholipid metabolism in glomerulosa cells. Second, viability of glomerulosa cells appears to be quite important to observe the PKC-dependent component of aldosterone output. In our hands, ANG II induces a more than 10-fold sustained increase in aldosterone production, but others do not find this [21]. This is particularly important for rat adrenal glomerulosa cells. As pointed out by Barrett et al. [25], it had been rather difficult to show that phorbol ester stimulates aldosterone production in rat glomerulosa cells. However, Vinson et al. [26] have shown that phorbol ester is in fact capable of stimulating aldosterone production in intact rat adrenal capsule, but not in collagenase-digested glomerulosa cells. Their data suggest that cell dispersion, probably by causing some damage to the cell surface, somehow abolishes phorbol ester-mediated aldosterone responses. In this regard, it is worth mentioning that, in adrenocortical cells, activation of PKC results in a release of the active lipid mediators 12-hydroxyeicosatetraenoic acid [9,27] and platelet-activating factor [28], which presumably modulate steroid production as autocrine factors. It is possible that release of those lipid mediators is impaired to some extent in collagenase-digested partially damaged cells. In any case, it is conceivable that involvement of PKC in ANG II action is rather difficult to demonstrate when using such phorbol ester-unresponsive cells.

In conclusion, ANG II induces sustained activation of PKC in adrenal glomerulosa cells, and Ca^{2+} entry is critical for the minute-to-minute regulation of the activity of PKC.

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