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CONTRASTING EFFECTS OF SN-1,2-DIOCTANOYL GLYCEROL AS COMPARED TO OTHER PROTEIN KINASE C ACTIVATORS IN ADRENAL GLOMERULOSA CELLS

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Summary—Angiotensin II acts on adrenal glomerulosa cells to induce the phospholipase C-mediated generation of inositol trisphosphate and sn-1,2-diacylglycerol as the major products of inositol phospholipid breakdown. This last product is known to activate protein kinase C, but its role in the action of angiotensin II on steroidogenesis has not been defined. We report herein that, in bovine adrenal glomerulosa cells, protein kinase C activators, such as phorbol 12,13-dibutyrate, 12-O-tetradecanoylphorbol-13-acetate, mezerein and sn 1,2 oleoyl acetoylglycerol, each failed to increase steroidogenesis. These results contrast with our recent report on the enhancement of aldosterone output by sn-1,2-dioctanoylglycerol (DiC8) [*J. Steroid Biochem.* **35** (1990) 19–33]. In addition, the difference between DiC8 and the other protein kinase activators was also observed in the pattern of ^{86}Rb efflux from preloaded glomerulosa cells; only DiC8 mimicked the effect of angiotensin II on ion fluxes. Furthermore, staurosporine, a potent inhibitor of protein kinase C, was capable of amplifying the aldosterone output induced by a maximally effective concentration of DiC8 or angiotensin II. These data suggest that the effect of the cell permeant DiC8 on aldosterone biosynthesis either is not mediated by protein kinase C activation, or is mediated by a phorbol ester-insensitive isoenzyme of protein kinase C.

INTRODUCTION

Angiotensin II induces rapid breakdown of polyphosphoinositide in adrenal glomerulosa cells. This reaction is catalyzed by phospholipase C to produce inositol-1,4,5-trisphosphate and the hydrophobic messenger 1,2-sn-diacylglycerol, the physiological activator of protein kinase C. Protein kinase C has been proposed to play a crucial role in the sustained phase of angiotensin II-induced aldosterone response [1]. This enzyme is activated by angiotensin II through redistribution of an inactive cytosolic form of the enzyme to the membrane [2–4]. However, the participation of protein kinase C in the steroidogenic effect of angiotensin II is controversial [5, 6]. The introduction of cell-permeable sn-1,2-diacylglycerol which competes for phorbol ester binding to and stimulates protein kinase C, has allowed researchers to study the role of protein kinase C using a more physiological stimulus. Recently, we have shown that sn-1,2-dioctanoylglycerol (DiC8) mimics the effects of angiotensin II on aldosterone production in glomerulosa cells [7]. Nevertheless, studies in other cell types have indicated that DiC8 may function in a stimulatory pathway that is independent of protein kinase C [8, 9]. Therefore, we decided to explore the possible involvement of protein kinase C in DiC8 mediated aldosterone biosynthesis, by comparing the pattern of its cellular activation with that of phorbol esters known as protein kinase C activators. Also, the role of protein kinase C in the steroidogenic action of angiotensin II and DiC8 was investigated by the use of staurosporine, a recently introduced protein kinase C inhibitor of great potency.

EXPERIMENTAL

Preparation of dispersed bovine adrenal glomerulosa cells

Dispersed adrenal glomerulosa cells were prepared from calves slaughtered at a local abattoir. The glands were freed of fat, and the glomerulosa zone was obtained by slicing thin

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sections from the outermost portions of the glands. The glomerulosa tissue was minced with scissors and washed with Krebs-Ringer–bicarbonate–glucose (KRBG) of the following composition: NaCl 115 mM, KCl 4 mM, $NaHCO_3$ 24 mM, NaH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 0.6 mM and glucose 11 mM. The solution was kept at pH 7.4 by gassing with 95% O_2 plus 5% CO_2.

The washed tissue was taken up into fresh medium containing 2 mg/ml bovine serum albumin and 2 mg/ml collagenase (Worthington Biochemical, St Louis, MO). The tissue was incubated at 37°C for 30 min under an atmosphere of 95% O_2–5% CO_2 in a shaking water bath. Isolated cells were obtained according to Fredlund *et al.* [10]. The viability of the cells was checked by their ability to exclude trypan blue (4%). After washing, the cells were incubated in KRBG supplemented with 2% albumin (fraction V, Sigma, St Louis, MO) throughout all the procedures (KRBGA).

Measurements of ^{86}Rb efflux

The procedure for measuring the ^{86}Rb efflux has been described previously [7]. Briefly, isolated bovine adrenal glomerulosa cells were incubated for 60 min at 37°C in KRBGA solution containing 1–2 μCi ^{86}Rb Cl/ml. At the end of this loading period, the cells were centrifuged at 500 *g* for 30 s and the supernatant discarded; the pellet was resuspended in a mixture of KRBGA and Sephadex G-10 (3:1). Aliquots of the cell suspension (equivalent to 2×10^6 cells) were placed in plastic syringes; each column contained a glass wool plug on which the cells with Sephadex were layered. The columns were placed in a water bath at 37°C and perfused with KRBGA through a Rainin miniature peristaltic pump. The flow rate through each column was 0.5 ml/min; the reservoir was gassed with 95% O_2–5% CO_2 and also kept at 37°C. The effluent from each chamber was collected directly into scintillation vials at intervals of 2 min, except for the first 20 min fractions that were discarded. At the end of each experiment, the cells were expelled from each syringe, homogenized and analyzed for the remaining radioisotope content. The radioactivity of ^{86}Rb was measured by Cerenkov radiation in a liquid scintillation counter. ^{86}Rb efflux was calculated from the release of radioactivity into the effluent media, assuming first-order kinetics, and that remaining in the cells at the end of the experiment [11].

$^{86}RbCl$ was purchased from the Chilean Atomic Energy Commission; angiotensin II and other drugs were obtained from Sigma or Calbiochem (San Diego, CA).

Measurement of aldosterone secretion

The time course of aldosterone secretion was determined from aliquots of the effluent in a similar protocol as the one described above for Rb fluxes. The aldosterone content was measured by radioimmunoassay as described previously [12]. The aldosterone production rate was expressed as picograms aldosterone per min per 10^6 cells.

Statistics

The results are expressed as mean ± SEM. Statistical comparisons were done by the Student's *t*-test or by analysis of variance when appropriate.

RESULTS

Comparative effect of different activators of protein kinase C on steroidogenesis

Isolated bovine adrenal glomerulosa cells were perfused in parallel chambers with a series of compounds related to protein kinase C activation. The following compounds were tested for their effect on aldosterone output: 12-O-tetradecanoylphorbol-13-acetate (TPA) (5–500 nM), sn 1,2 oleoylacetoylglycerol (OAG) (5–50 μM), phorbol 12,13-dibutyrate (DiBu) (50–500 nM), Mezerein (1 μM), 1-monooleyl-rac-glycerol (MOG) (1 μM) and DiC8 (3–300 μM).

Of these compounds, only DiC8 had a significant stimulatory effect on steroidogenesis. Figure 1 includes the results obtained with the highest concentration of the above indicated drugs. As shown in the figure, when DiC8 was added to the perfusate, there was a rapid increase in aldosterone secretory rate; however the other protein kinase C activators failed to significantly increase aldosterone output. The effect of these compounds in the presence of a calcium ionophore (A23187 or ionomycin) was tested, since it has been shown that protein kinase C activators require the presence of calcium. As shown previously by us and others [7, 13, 14], the ionophore caused a small and transient increment in aldosterone secretion. When both, calcium ionophore and protein kinase activators were added together, there was no further increment of aldosterone secretion (results not shown).

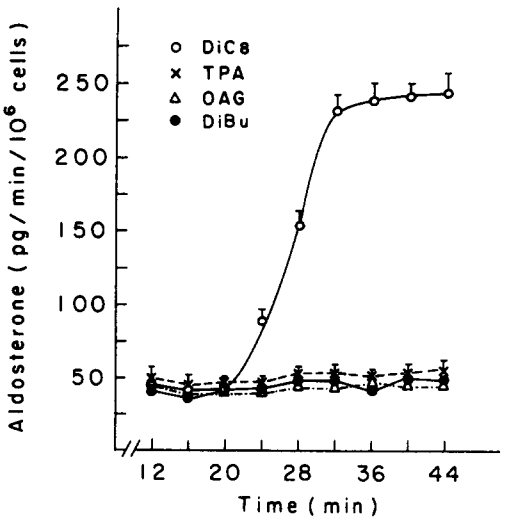

Fig. 1. Comparison of time course of aldosterone secretory response to DiC8, TPA, OAG and DiBu. Bovine adrenal glomerulosa cells were perfused in parallel columns with KRBG containing 0.6 mM Ca and 2% bovine serum albumin (BSA) for an equilibration period of 30 min. At time 0 control samples were collected for aldosterone measurements during 20 min. At 20 min, either 300 μM DiC8 (o) 100 nM TPA (x), 60 μM OAG (△) or 10 nM DiBu (●) was added to perfusate. Aldosterone output is expressed as mean values of the absolute rate of production per 10^6 cells ($n = 5$).

Effect of DiC8, OAG and TPA on potassium permeability

We have previously shown that ^{86}Rb efflux pattern is a measurement of potassium permeability of the adrenal cells [12]. Therefore, the rate of ^{86}Rb efflux from bovine adrenal glomerulosa cells was measured in the presence of different activators of protein kinase C. Figure 2 illustrates the changes which occurred in the efflux of ^{86}Rb when cells preloaded with the radioisotope were exposed to 300 μM DiC8, 60 μM OAG or 100 nM TPA. As shown in Fig. 2, the three agents produce a significant increment in the coefficient rate of ^{86}Rb efflux. The increased ^{86}Rb efflux was transient. Extending the time course of those experiments reveals that the increase in ^{86}Rb efflux mediated by DiC8 was followed by a second phase in which an inhibition of the efflux was observed, whereas such inhibitory phase was not observed with the two other compounds: OAG or TPA.

Effect of DiC8, OAG and TPA on ^{86}Rb efflux in depolarized glomerulosa cells

In previous studies we have found that increasing external potassium from 4 mM up to 12 mM K^+ produces a significant increment on the rate coefficient of ^{86}Rb efflux from adrenal glomerulosa cells. Further, we demonstrated that the enhancement on Rb efflux due to depolarization was abolished by the presence of angiotensin II or DiC8 [7, 12]. Therefore we decided to compare the effect of DiC8, OAG and TPA on potassium-mediated ^{86}Rb efflux. The results are shown in Fig. 3. Cells preloaded with ^{86}Rb were perfused with a Krebs-Ringer solution (KRBGA) containing 4 mM K and 300 μM DiC8, or 60 μM OAG or 100 nM TPA were added 20 min before the potassium-induced depolarization. As shown in Fig. 3, the increment in the coefficient rate of ^{86}Rb efflux due to potassium depolarization was significantly reduced ($P < 0.01$) in the presence of 300 μM DiC8 to values near the control levels (see Fig. 2). In contrast, an enhancement on ^{86}Rb efflux was observed in the presence of 100 nM TPA in the depolarized cells. No significant effect was observed with OAG on the potassium-mediated ^{86}Rb efflux. Other compounds were also tested: DiBu (500 nM), MOG (1 μM) and mezerein (1 μM) but these compounds did not affect the potassium-mediated ^{86}Rb efflux (results not shown).

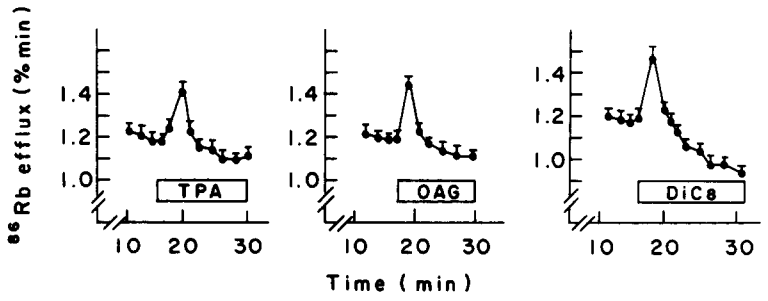

Fig. 2. Effect of TPA, OAG and DiC8 on the rate coefficient of ^{86}Rb efflux from perfused bovine adrenal glomerulosa cells. In this and subsequent experiments, glomerulosa cells were preequilibrated with ^{86}Rb for 60 min. Cells were transferred into perfusion chambers and perfused with KRBG containing 0.6 mM Ca and 2% BSA during 20 min before starting sample collection. Protein kinase C activators were added at 14 min: 100 nM TPA, 60 μM OAG or 300 μM DiC8. Each point is the mean value ± SE of 4 separate experiments.

Effect of staurosporine on aldosterone output

Staurosporine has been shown to be a potent inhibitor of protein kinase C [15]. Therefore, we decided to study the effect of this alkaloid on the steroidogenic effect of DiC8 and angiotensin II to further test whether protein kinase C plays a crucial role in signal transduction in response to these two agents. Cells were perfused with staurosporine 8 min before the addition of the stimulus. The results of three different experiments are shown in Fig. 4; mean values were calculated with the maximal stimulus obtained under the different conditions indicated in the figure. As shown, 100 nM staurosporine itself produced an increment in aldosterone production. Staurosporine not only failed to inhibit either angiotensin II or DiC8 stimuli on steroidogenesis, but significantly enhanced the secretory action of both agents ($P < 0.05$).

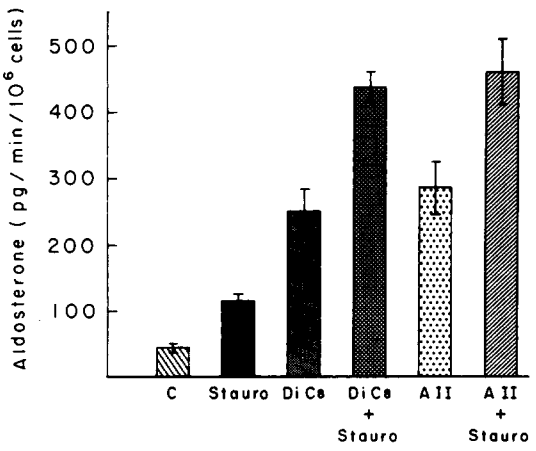

Fig. 4. Effect of staurosporine on aldosterone production. Isolated bovine adrenal glomerulosa cells were perfused in parallel chambers with or without staurosporine (100 nM) and DiC8 (100 μM) or angiotensin II (100 nM). The bars represent mean values ± SE of the maximal stimulus obtained under the different conditions. C = control; Stauro = staurosporine; AII = angiotensin II.

DISCUSSION

Cell-permeant DiC8 was previously shown by us to stimulate aldosterone biosynthesis in isolated bovine adrenal glomerulosa cells, either in the presence or absence of a calcium-ionophore. In the present study we demonstrate that the stimulation of aldosterone production elicited by DiC8 was not mimicked by other well known activators of protein kinase C such as OAG and tumor promoters TPA, DiBu, mezerein, etc, when these compounds were used at a concentration maximally effective in other systems. The absence of an effect of phorbol esters on aldosterogenesis suggests that the response is not mediated by activation of protein kinase C. Since there are different forms of protein kinase C, however, one cannot rule out the possibility that the effect of DiC8 is mediated by a protein kinase C-like protein that is not stimulated by phorbol esters [16, 17].

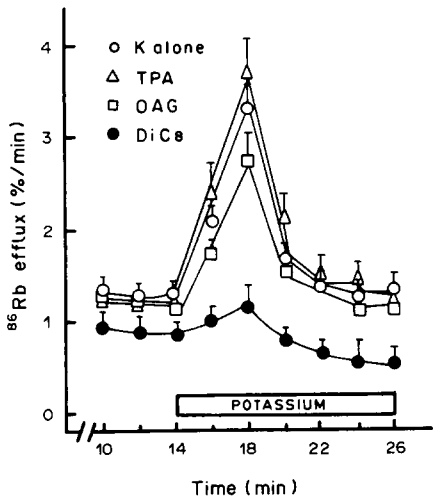

Fig. 3. Effect of TPA, OAG and DiC8 on potassium stimulated ^{86}Rb efflux from perfused glomerulosa cells. Preloaded ^{86}Rb cells were mounted in columns and at the end of the equilibration period, protein kinase C activators were added to the perfusion solution. At 14 min, 12 mM K was added to the perfusate: 12 mM K (○), 12 mM K plus 100 nM TPA (△), 12 mM K plus 60 μM OAG (□) and 12 mM K plus 300 μM DiC8 (●). Each point is the mean ± SE of 5 experiments.

Nakano *et al.* [6] presented evidence against the involvement of protein kinase C in the stimulation of aldosterone production by angiotensin II and Spat *et al.* [18] failed to observe a stimulation by TPA of aldosterone secretion in rat glomerulosa cells. In the present study we have been able to investigate more directly this problem by the use of staurosporine. This compound has been shown to be a potent inhibitor of protein kinase C in several tissues [15]. When we examined the effect of staurosporine on the angiotensin II or DiC8-mediated steroidogenesis, we found that aldosterone response to these secretagogues was not only preserved but enhanced, supporting the notion that activation of protein kinase C is not necessary for the steroidogenic action of angiotensin II. These results are the first demonstration of an enhancement by staurosporine of steroidogenesis in adrenal glomerulosa cells.

Further, we investigated the effect of protein kinase C activators on potassium permeability since previous work has shown a biphasic effect

of DiC8 and angiotensin II on ^{86}Rb efflux: an initial increment followed by a diminished efflux [7, 12]. The present results indicate that TPA and OAG produced a significant increment on potassium permeability, but this effect was not followed by the inhibitory phase. Further, all the compounds tested failed to block the increment in potassium permeability mediated by depolarization of the glomerulosa cells, as observed with DiC8. These data support the hypothesis that the inhibition of plasma membrane potassium permeability is important for the steroidogenic response [19]. Taken together all the present data would suggest that the effect of DiC8 on potassium permeability is not mediated by activation of protein kinase C.

It is possible that the more permeable DiC8 can be rapidly metabolized and converted to the corresponding phosphatidic acids and probably further to inositol phospholipids [20, 21]. Arachidonic acid itself, or some derived eicosanoids could be involved in the response of adrenal glomerulosa cells to DiC8 [22].

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