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SN-1,2 DIOCTANOYLGLYCEROL MIMICS THE EFFECTS OF ANGIOTENSIN II ON ALDOSTERONE PRODUCTION AND POTASSIUM PERMEABILITY IN ISOLATED BOVINE GLOMERULOSA CELLS

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Summary—In order to elucidate the possible role in glomerulosa cells of diacylglycerol released by angiotensin II we have studied the action of a synthetic diacylglycerol, sn-1,2-dioctanoylglycerol (DiC8), on aldosterone production and potassium permeability in bovine adrenal cells. DiC8 elicited an increase in ^{86}Rb efflux from cells previously equilibrated with the isotope. The action of DiC8 on the rate coefficient for ^{86}Rb efflux was similar to that previously described for angiotensin II (*Am. J. Physiol.* **254** (1988) E144–149), i.e. DiC8 induced an immediate increase in ^{86}Rb efflux followed by a sustained decrease in potassium permeability. This DiC8 induced inhibition was observed even in the presence of depolarizing concentrations of potassium.

The effect of DiC8 on aldosterone secretion from adrenal glomerulosa cells was measured using a perifusion system. DiC8 (300 μM) caused a signifïcnt increase of aldosterone production, comparable to that seen with angiotensin II (100 nM). These results indicate that DiC8 has similar effects to angiotensin II on both potassium permeability and steroidogenesis, which suggests that activation of protein kinase C is involved in the changes of ionic permeability induced by this hormone in bovine adrenal glomerulosa cells.

INTRODUCTION

Bovine adrenocortical glomerulosa cells respond to several effectors with increased steroidogenic activity. Angiotensin II and external potassium are of major physiological significance. Angiotensin II has been shown to activate adrenocortical glomerulosa cell phosphatidylinositol (PI) turnover. Phosphoinositide breakdown products such as diacylglycerol (DAG) and inositol, 4,5-trisphosphate (IP_3) mediated Ca released have been suggested as intracellular messengers acting through the activation of a Ca activated phospholipid dependent protein kinase C [1].

Diacylglycerols containing a sn-1,2 configuration with various fatty acids of different chain length are capable of activating protein kinase C, with those having an unsaturated fatty acid being most active [2, 3]. Recently, 1,2 dioctanoylglycerol and 1,2 didecanoglycerol was shown to be cell-permeable [4]. Thus, these diacylglycerols are often used in studies to explore possible roles of the enzyme in stimulus response coupling.

The present study was initiated to investigate the biochemical mechanisms underlying the response to angiotensin II by examining the functional significance of one of the metabolites of the phosphoinositide pathway, DAG, the second messenger that activates protein kinase C [1]. We have studied the effect of one activator of protein kinase C, namely DiC8.

The present results indicate that DiC8 is a potent stimulus of steroidogenesis and modifies ionic fluxes in bovine adrenal glomerulosa cells. Both effects are similar to those observed in response to angiotensin II.

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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 sections from the outermost portions of the gland. The glomerulosa tissue was briefly minced with scissors and washed with Krebs–Ringer–bicarbonate–glucose (KRBG) of the following composition (mM): NaCl 115, KCl 4, $NaHCO_3$ 24, NaH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 0.6 and glucose II. The solution was maintained 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.*[5]. 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) throughout all the procedures (KRBGA).

Measurements of ^{86}Rb efflux

The procedure for measuring the ^{86}Rb efflux has been described previously [6]. Briefly, isolated bovine adrenal glomerulosa cells were incubated for 60 min at 37°C in KRBG solution containing 1–2 μCi ^{86}Rb. 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. 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 perifused 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. After 20 min, the effluent from each chamber was collected directly into scintillation vials at intervals of 2 min. At the end of the experiment, the cells were expelled from each syringe, homogenized and analyzed for the remaining radioactivity. The radioactivity of ^{86}Rb was measured in a liquid scintillation counter relying on Cerenkov radiation. ^{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 [7].

The ^{86}RbCl was purchased from the Chilean Atomic Energy Commission; angiotensin II and other drugs were obtained from Sigma.

Measurement of aldosterone secretion

The time-course of aldosterone secretion was determined from aliquots of effluent the protocol was similar to that described above for ^{86}Rb fluxes. The aldosterone content was measured by radioimmunoanalysis as described previously [8]. The aldosterone production rate was expressed as picograms aldosterone per min per 10^6 cells.

RESULTS

Comparative effect of DiC8, ionomycin and angiotensin II on aldosterone production

The secretory responses of isolated bovine adrenal glomerulosa cells perfused in parallel chambers, with 300 μM DiC8 or 100 nM angiotensin II are shown as a function of time in Fig. 1. When DiC8 is added to the perifusate, there is a rapid increase in aldosterone secretory rate reaching a maximum at 20 min (Fig. 1A). The magnitude of the response is similar to that obtained in the presence of 100 nM angiotensin II (Fig. 1B).

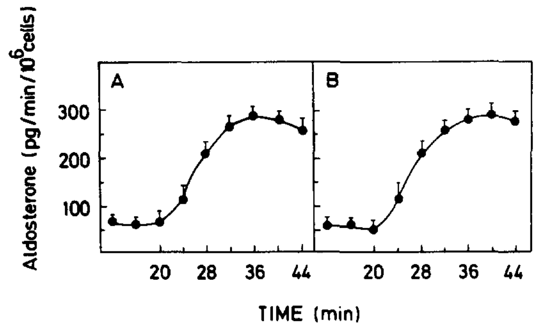

Fig. 1. Comparison of the time-course of aldosterone secretory response to DiC8 and angiotensin II. Bovine adrenal glomerulosa cells were perifused in parallel columns with KRBG containing 0.6 nM Ca and 2% BSA for an equilibration period of 30 min. After equilibration (time 0), control samples were collected for aldosterone measurements during 20 min. At 20 min, either 300 μM DiC8 (A), or 100 nM angiotensin II (B) was added to perifusate. Aldosterone output was expressed as rate of production: pg/min per 10^6 cells. Mean values ± SE of 6 experiments.

The effect of DiC8 in the presence of a calcium ionophore was tested since it has been shown that protein kinase C activators require the presence of calcium for translocation of the enzyme from the cytosol to the particulate fraction [9, 10].

When 2 μM ionomycin is added to the perifusate, there is a small increment in aldosterone secretion starting at about 10 min after the addition of the ionophore, which reaches only about twice the basal value; this rate then declines to control values. When both, DiC8 and ionomycin are added together, the temporal pattern of the aldosterone secretory response is similar to that seen after addition of 100 nM angiotensin II (see Fig. 2). In this last condition, aldosterone secretion reaches a plateau and then stays at this value.

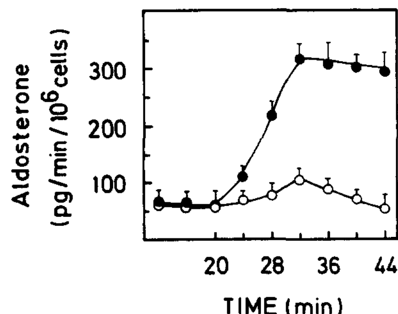

Fig. 2. Effect of DiC8 on aldosterone output in the presence of ionomycin. Conditions used throughout the experiment were as described in Fig. 1. At 20 min, DiC8 (300 μM) plus ionomycin (2 μM) were added simultaneously (●). Cells in parallel columns received ionomycin (2 μM) alone (○). Mean values ± SE of 5 experiments.

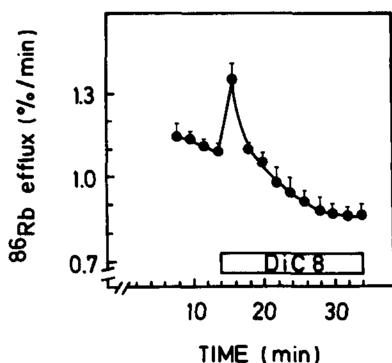

Fig. 3. Effect of DiC8 on the rate coefficient of ^{86}Rb efflux from perifused bovine adrenal glomerulosa cells. In this and subsequent experiments, glomerulosa cells were preequilibrated with ^{86}Rb for 60 min. Cells were transferred into perifusion chambers and perifused with KRBG containing 0.6 mM Ca and 2% BSA for 20 min before starting sample collection. DiC8 (100 μg/ml) was added at 14 min. Mean values ± SE of 4 separate experiments.

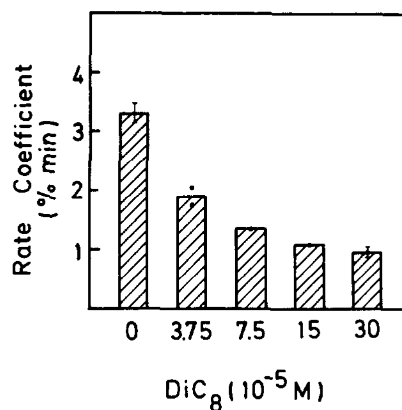

Fig. 5. Effect of different concentrations of DiC8 on potassium stimulated ^{86}Rb efflux. Cells preloaded with ^{86}Rb were perifused with DiC8 for 20 min before raising the potassium from 4 to 12 mM K. The columns represent mean values (2–4 experiments) of the maximal rate constant for ^{86}Rb efflux achieved with the potassium stimulus.

Effect of DiC8 on ^{86}Rb efflux in glomerulosa cells

The rate of ^{86}Rb efflux from bovine adrenal glomerulosa cells was measured in the presence of DiC8, a synthetic analog of diacylglycerol. Figure 3 illustrates the changes which occurred in ^{86}Rb efflux when cells preloaded with ^{86}Rb are exposed to 300 μM DiC8. As shown in Fig. 3, this agent produces a significant increase in the rate coefficient for ^{86}Rb efflux. The increment of ^{86}Rb efflux is transient. Extending the time-course of these experiments reveals that the increase in ^{86}Rb efflux mediated by DiC8 is followed by a second phase in which inhibition of the efflux is observed.

In previous studies, we have shown that angiotensin II modifies potassium permeability with a pattern similar to those observed in Fig. 3. The angiotensin II mediated inhibitory phase on ^{86}Rb efflux was observed even during depolarization of the cells, or in the presence of a calcium ionophore [11]. Therefore, we have examined the effect of an increment of external K (from 4 to 12 mM) in cells treated with DiC8 or angiotensin II. The results are summarized in Fig. 4. Cells preloaded with ^{86}Rb were perifused with a Krebs–Ringer solution containing 4 mM K and 300 μM DiC8, or 100 nM angiotensin II were added 20 min before the potassium stimulus (12 mM K). As shown in the figure, the increment in the rate coefficient for ^{86}Rb efflux due to potassium depolarization was significantly reduced in the presence of DiC8 to values near those observed in the presence of angiotensin II.

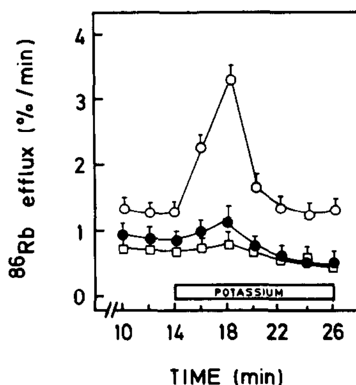

Fig. 4. Effect of DiC8 and angiotensin II on potassium stimulated ^{86}Rb efflux from perifused glomerulosa cells. Preloaded ^{86}Rb cells were mounted in columns and DiC8 or AII were added to the perifusion solution 6 min before starting sample collection. At 14 min, potassium was raised from 4 to 12 mM K in the perifusate: 12 mM K (○), 12 mM K plus 300 μM DiC8 (●), and 12 mM K plus angiotensin II (□). Mean values ± SE of 5 experiments.

The inhibitory effect of DiC8 on potassium-mediated ^{86}Rb efflux was further investigated at different concentrations of this compound. The results at four concentrations of DiC8 are shown in Fig. 5. As indicated in the figure DiC8 blocked the increment in ^{86}Rb efflux due to 12 mM K in a concentration-dependent manner.

DISCUSSION

Diverse biochemical and cellular changes occur in response to extracellular agents that stimulate phosphatidylinositol hydrolysis, mobilization of Ca^{2+}, and activation of a lipid-dependent protein kinase termed protein kinase C. Diacylglycerols produced from phosphatidylinositols are naturally occurring activators of protein kinase C. Some synthetic diacylglycerols, such as 1-oleoyl-2-acetylglycerol, readily intercross intact cell membranes and activate protein kinase C directly [4]. Recently, sn-1,2-dioctanoylglycerol (DiC8) was shown to be membrane permeable [3]. Thus this compouind can be used to explore possible roles of the enzyme in stimulus-response coupling. In this paper we provide evidence that in adrenal glomerulosa cells 1,2-dioctanoylglycerol

enhances aldosterone secretion to levels similar to those observed in the presence of maximal angiotensin II concentration.

A significant effect of DiC8 on steroidogenesis was observed even in the absence of a Ca ionophore. Although some previous studies with phorbol 12-myristate 13-acetate (TPA) or 1-oleoyl-2-acetyl-glycerol (OAG) indicated that a calcium-ionophore was required in order to obtain striking increment of aldosterone output in bovine adrenal cells [12, 13], others have been unable to obtain any stimulus in rat glomerulosa cells [14, 15]. Vinson *et al.* have shown that TPA is able to stimulate aldosterone output only in intact rat capsule preparations [15]. Diacylglycerol analogs and phorbol esters are often assumed to have identical effects; however, this is not always the case [16, 17]. In fact, Nishisuka[1] mentions that differences in the actions of phorbols and DG analogs should not be surprising. The cells-permeable diacylglycerol, DiC8, has been shown to mimic the effect of tumor promoting phorbol diesters in several tissues [3, 18] by activating protein kinase C directly. It is conceivable that with the permeable analog the importance of an increase of cellular calcium is less significant and DiC8 is able to increase aldosterone output to values similar to angiotensin II even in the absence of a calcium ionophore. In adrenal glomerulosa cells, protein kinase C is activated by angiotensin II [9, 10] and it has been postulated by Rasmussen *et al.* that this activation is involved in the sustained phase of the hormone-mediated steroidogenesis [19].

In some cells activation of protein kinase C is linked to depolarization through blockage of potassium channels [20–22] and DiC8 has been shown to inhibit depolarization-induced ^{86}Rb efflux [22]. In bovine adrenal glomerulosa cells, we have shown a sustained inhibition on ^{86}Rb efflux under the action of angiotensin II. In fact, this polypeptide hormone induces a transient increase in the rate coefficient of isotope efflux (^{86}Rb or ^{42}K) followed by a reduction in potassium permeability [11]. The present results indicate that DiC8 not only mimics the steroidogenic effect of angiotensin but also produces a similar biphasic change in ^{86}Rb efflux.

Whether or not the effect of DiC8 is mediated through activation of protein kinase C requires further studies. 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 [23]. On the other hand, it has been shown that DiC8 inhibits lysophosphatide acyltransferase [24]. In macrophages it has been shown that significant arachidonic acid release was only detectable when the reacylating enzyme, lysophosphatide acyltransferase was inhibited. Arachidonic acid itself, or some derived eicosanoids could be involved in the response of adrenal glomerulose cells to DiC8. As suggested by others eicosanoids (arachidonic-acid metabolites) can be added to the list of second messengers [25].

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