Phorbol ester inhibits angiotensin-induced activation of phospholipase C in adrenal glomerulosa cells

Its implication in the sustained action of angiotensin

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The present study was undertaken to determine whether an agonist-induced activation of C-kinase leads to an inhibition of phospholipase C in adrenal glomerulosa cells. When cells are treated with 100 nM-TPA (12-*O*-tetradecanoylphorbol 13-acetate), subsequent angiotensin ('angiotensin II')-induced aldosterone secretion is greatly inhibited. Treatment with TPA completely inhibits the angiotensin-induced increase in both inositol trisphosphate and the cytosolic Ca^{2+} concentration. The dose-response curve for TPA-induced inhibition reveals that quite a high concentration of TPA is necessary to block angiotensin action compared with that needed to stimulate aldosterone secretion. 1-Oleoyl-2-acetyl-glycerol has a weak inhibitory effect, whereas neither 4 α -phorbol 12,13-didecanoate or 4 β -phorbol inhibits angiotensin action. When the time course of changes in inositol trisphosphate and diacylglycerol is measured, angiotensin action is sustained for up to 30 min. In addition, 100 nM-TPA added after 20 min of angiotensin addition attenuates production of both inositol trisphosphate and diacylglycerol. These results suggest that high dose of TPA inhibits angiotensin-induced activation of phospholipase C by acting, at least partly, on C-kinase, but that an inhibitory effect of TPA may be a pharmacological effect with little physiological significance in this system.

INTRODUCTION

Recent progress in the study of the calcium-messenger system has provided many insights into the action of Ca^{2+} -mobilizing agonists. An early event after binding of the agonist to its receptor is an activation of phospholipase C specific to $\text{PtdIns}(4,5)\text{P}_2$ leading to the generation of two products, each of which has unique messenger function (Berridge, 1984). Inositol trisphosphate mobilizes Ca^{2+} from the intracellular non-mitochondrial pool (Berridge & Irvine, 1984) and another product, diacylglycerol, acts as a sensitivity modulator of a unique Ca^{2+} -dependent enzyme, protein kinase C (Nishizuka, 1984). The roles of these two messengers were first demonstrated in platelets by the use of a bivalent-cation ionophore (A23187) to activate the Ca^{2+} -calmodulin branch and a phorbol ester, TPA, to activate the C-kinase branch of the calcium-messenger system (Yamanishi, *et al.*, 1983). When both the Ca^{2+} -calmodulin branch and the C-kinase branch are activated simultaneously by a combination of ionophore A23187 and TPA, the resultant 5-hydroxytryptamine (serotonin) secretion is comparable with that stimulated by a natural agonist, thrombin, suggesting that thrombin activates both branches of the calcium-messenger system. Subsequently we have extended this model in finding that the Ca^{2+} -calmodulin branch is mainly responsible for an initial cellular response and the C-kinase branch for sustained response (Kojima *et al.*, 1983). The two-branch

model has been validated for many secretory cells (Rasmussen & Barrett, 1984).

Recently, an additional role for C-kinase, namely the inhibition of agonist-induced activation of phospholipase C, has been suggested in some systems: in platelets and neutrophils, pretreatment with TPA completely abolishes agonist-induced hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ and elevation of the cytosolic Ca^{2+} concentration $[\text{Ca}^{2+}]_c$ (Watson & Lapetina, 1985; Zavoico *et al.*, 1985; Naccache *et al.*, 1985). These results have been interpreted simply to indicate that the activation of C-kinase terminates agonist-induced hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$. The physiological relevance of this TPA-induced inhibition of agonist action, however, has not been evaluated critically. In many systems, Ca^{2+} -mobilizing agonists induce sustained cellular responses (Rasmussen *et al.*, 1984). It is not clear whether such agonists terminate their own actions by activating C-kinase in those systems.

The purpose of the present study was to evaluate the physiological significance of TPA-induced inhibition of agonist action by using the adrenal glomerulosa cell as a model system. In this system, angiotensin ('angiotensin II') causes sustained aldosterone secretion by activating both the Ca^{2+} -calmodulin and C-kinase branches of the calcium-messenger system (Kojima *et al.*, 1984). Results indicate that angiotensin causes sustained activation of phospholipase C and that TPA-induced inhibition may be a pharmacological effect with little physiological significance.

Abbreviations used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; $\text{PtdIns}(4,5)\text{P}_2$, phosphatidylinositol 4,5-bisphosphate; $\text{PtdIns}4\text{P}$, phosphatidylinositol 4-phosphate; $[\text{Ca}^{2+}]_c$, cytosolic concentration of Ca^{2+} ; Me_2SO , dimethyl sulphoxide; 4 α -PDD, 4 α -phorbol 12,13-didecanoate; OAG, 1-oleoyl-2-acetyl-glycerol.

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MATERIALS AND METHODS

Materials

Angiotensin, carbamylcholine, atropine, TPA, 4 α -PDD and 4 β -phorbol were purchased from Sigma (St. Louis, MO, U.S.A.). OAG was a gift from Professor Y. Nishizuka of Kobe University, Kobe, Japan. [^3H]Inositol (15.8 Ci/mmol) was obtained from New England Nuclear Corp. (Boston, MA, U.S.A.).

Preparation and perfusion of adrenal glomerulosa cells

Bovine adrenal glomerulosa cells were prepared by collagenase digestion (Kojima *et al.*, 1983). Cells were resuspended in modified Hanks solution containing (mM): NaCl, 137; KCl, 3.5; KH_2PO_4 , 0.4; NaHCO_3 , 4.2; Na_2HPO_4 , 0.33; CaCl_2 , 0.5; Hepes (pH 7.4), 20; and glucose, 5.5 (equilibrated with O_2). Perfusion was performed as described previously (Delbeke *et al.*, 1984) with the flow rate of 0.4 ml/min. Samples for aldosterone were collected every 5 min and aldosterone was measured by radioimmunoassay (Ogihara *et al.*, 1977).

TPA, 4 α -PDD, 4 β -phorbol and OAG were dissolved in Me_2SO and the final concentration of Me_2SO never exceeded 0.3%, a concentration that had no effect on aldosterone secretion.

Measurement of [^3H]inositol phosphates and diacylglycerol

Production of inositol phosphates was measured by using [^3H]inositol-labelled glomerulosa cell. Adrenal glomerulosa cells were incubated for 2 h with 10 μCi of [^3H]inositol/ml and 1 μM -carbamylcholine. At 5 min before the end of labelling period, 50 μM -atropine was added to terminate the action of carbamylcholine. After a 2 h labelling period, cells were washed twice with modified Hanks solution containing 10 mM-inositol. This procedure was as effective as labelling with a low dose of angiotensin followed by blockade with angiotensin antagonist (Kojima *et al.*, 1984). A cell suspension containing 4×10^6 cells was stimulated for the indicated time in the absence of Li^+ and the reaction was stopped by the addition of chloroform/methanol (1:2, v/v). Inositol phosphates were extracted under acidic conditions as described previously (Kojima *et al.*, 1984) and were separated by anion-exchange column chromatography by the method of Berridge *et al.* (1983).

For measurement of diacylglycerol, a cell suspension containing 3×10^7 cells was stimulated for the indicated time and the reaction was stopped by adding chloroform/methanol. Diacylglycerol was measured by the method of Banschbach *et al.* (1974).

Measurement of $[\text{Ca}^{2+}]_i$

$[\text{Ca}^{2+}]_i$ was measured by using aequorin by the method of Morgan & Morgan (1982), with slight modification. Aequorin was loaded into the cells by making the plasma membrane reversibly permeable; this was done by incubating the cells for 20–40 min sequentially in the following solutions at 4 °C. The compositions of the solutions were as follows (mM). Solution I: EGTA, 10; Na_2ATP , 5; KCl, 120; MgCl_2 , 2; and Hepes (pH 7.1), 20; solution II: EGTA, 0.1; Na_2ATP , 5; KCl, 120; MgCl_2 , 2; Hepes (pH 7.1), 20; and aequorin (0.2 mg/ml); solution III: EGTA, 0.1; Na_2ATP , 5; KCl, 120; MgCl_2 , 10; and Hepes (pH 7.1), 20. Cells were then resuspended in a solution [containing (mM): NaCl, 120; KCl, 3.5;

NaHCO_3 , 5.5; MgCl_2 , 10; NaH_2PO_4 , 1.4; Hepes (pH 7.4), 20; and glucose, 5.5] and incubated at room temperature for 60 min. Cells were then washed and resuspended in modified Hanks solution. The aldosterone response in aequorin-loaded cells is identical with that in unloaded cells. Furthermore, it was possible to maintain aequorin-loaded cells in primary culture for at least a week. The aequorin signal was measured by using a Chronolog platelet aggregometer (Havertown, PA, U.S.A.) with constant stirring at 37 °C (Johnson *et al.*, 1985). The resting $[\text{Ca}^{2+}]_i$ was calibrated by lysing unstimulated cell with Triton X-100, assuming the intracellular concentration of Mg^{2+} to be 1 mM (Blinks *et al.*, 1982; Johnson *et al.*, 1985). $[\text{Ca}^{2+}]_i$ in stimulated cell was not calibrated. Traces presented are the representative of five experiments giving similar results.

RESULTS

Effect of TPA pretreatment of angiotensin-induced aldosterone secretion

When 1 nM-angiotensin was added to the perfusion medium, the aldosterone secretion rate increased quickly after a 5 min interval. The secretory response is monotonic, and the secretion rate remains elevated as long as angiotensin is present. When glomerulosa cells were pretreated with 100 nM-TPA for 10 min and 1 nM-angiotensin was added in the presence of TPA, angiotensin-induced aldosterone secretion was greatly inhibited (Fig. 1). This TPA-induced inhibition was not overcome by adding higher dose of angiotensin (results not shown). Fig. 2 depicts the dose-response curve for TPA-induced inhibition of angiotensin-stimulated aldosterone secretion. TPA-induced inhibition is observed at 50 nM and is maximum at 100 nM. To examine whether TPA exerts its action by activating C-kinase, the inhibitory actions of 4 β -phorbol, 4 α -PDD and OAG were evaluated. As Table 1 shows, neither 4 β -phorbol or 4 α -PDD inhibited the effect of angiotensin. On the other hand, 120 μM -OAG had a small inhibitory effect on angiotensin-mediated secretion.

Effect of TPA pretreatment on angiotensin-induced activation of phospholipase C

To examine the effect of TPA on angiotensin-induced activation of phospholipase C, the production of [^3H]inositol phosphates was determined by using [^3H]inositol-labelled glomerulosa cells (Fig. 3). Angiotensin causes an immediate increase in [^3H]inositol trisphosphate and in bisphosphate within 20 s. [^3H]inositol monophosphate does not change within 60 s. When cells are pretreated with 100 nM-TPA for 10 min, subsequent angiotensin action on inositol phosphates is completely abolished.

Effect of TPA treatment on angiotensin II-induced changes in $[\text{Ca}^{2+}]_i$

In a previous study we showed that angiotensin-induced mobilization of Ca^{2+} from an intracellular pool is mediated by inositol trisphosphate (Kojima *et al.*, 1984). To characterize further the TPA action, we measured changes in $[\text{Ca}^{2+}]_i$ by using the Ca^{2+} -sensitive photoprotein aequorin. As Fig. 4 shows, addition of angiotensin results in an immediate increase in the bioluminescence of aequorin.

Fig. 1. Time course of angiotensin-induced aldosterone secretion and effect of TPA treatment

Perfusion was done as described in the Materials and methods section. Control cells (●) were stimulated from 0 to 60 min by 1 nM-angiotensin. Cells pretreated for 10 min with 100 nM-TPA (○) were stimulated from 0 to 60 min by 1 nM-angiotensin in the presence of 100 nM-TPA. Values are means $\pm$ S.E.M. for three experiments.

Fig. 2. Dose-response curve for TPA-induced inhibition of angiotensin-stimulated aldosterone secretion

Cells were pretreated with various doses of TPA for 10 min and then stimulated by 1 nM-angiotensin in the presence of TPA as described in the legend to Fig. 1. Aldosterone secreted from 0 to 60 min in response to 1 nM-angiotensin was compared with that in TPA-untreated cells. TPA-induced inhibition is expressed as a percentage of aldosterone secreted in cells not treated with TPA. Values are means for three to four experiments.

Table 1. Effect of pretreatment with OAG, 4 α -PDD and 4 β -phorbol on angiotensin-stimulated aldosterone secretion

Cells were pretreated with the given compound for 10 min and stimulated by 1 nM-angiotensin as described in the legend to Fig. 1. Aldosterone secreted from 0 to 60 min was compared with that in non-pretreated cells. Values are means $\pm$ S.E.M. for three experiments.

Pretreatment	Inhibition (%)
OAG (120 μ M)	18.1 $\pm$ 6.1
4 α -PDD (100 nM)	1.0 $\pm$ 6.6
4 β -Phorbol (100 nM)	2.1 $\pm$ 4.9

Fig. 3. Effect of TPA pretreatment on angiotensin-induced changes in inositol phosphates

Cells were labelled with [3 H]inositol as described in the Materials and methods section. Either control cells (○) or cells pretreated with 100 nM-TPA for 10 min (●) were stimulated for the indicated time by 1 nM-angiotensin in the absence of Li $^+$. Values are means $\pm$ S.E.M. for four experiments. Abbreviations: InsP $_3$, inositol trisphosphate; InsP $_2$, inositol bisphosphate; InsP, inositol phosphate.

A sharp peak is obtained within 5 s. The light emission then quickly decreases to a plateau that is slightly higher than the basal level, and remains there for about 1 min. At 2 min after the addition of angiotensin, the light emission is almost identical with the basal level. The transient nature of the aequorin signal is not due to the

Fig. 4. Effect of TPA pretreatment on angiotensin (ANG)-induced changes in $[Ca^{2+}]_i$.

Aequorin was loaded into glomerulosa cells as described in the Materials and methods section. Either control cells (left) or cells pretreated with 100 nM-TPA for 10 min (right) were stimulated by 1 nM-angiotensin. Traces are representative of five experiments with similar results. $[Ca^{2+}]_i$ in the resting cell was 146 ± 32 nM (mean $\pm$ S.E.M., $n = 6$).

consumption of aequorin, since subsequent addition of 8 mM- K^+ induces an increase in light emission (results not shown). Furthermore, the amount of aequorin remaining in the cell (estimated by addition of Triton X-100) is not significantly changed after stimulation with angiotensin (results not shown). When 100 nM-TPA is added, there is no change in the light emission; however, subsequent angiotensin-induced increase in light emission is completely blocked.

Time course of changes in inositol trisphosphate and diacylglycerol in angiotensin-stimulated cells

Results shown in Fig. 3 indicate that TPA treatment abolishes subsequent angiotensin-induced activation of $PtdIns(4,5)P_2$ - and possibly $PtdIns4P$ -specific phospholipase C. Furthermore, TPA seems to act by stimulating C-kinase. We showed previously that angiotensin acts by stimulating both the Ca^{2+} -calmodulin and the C-kinase branches of the calcium-messenger system. The question then arises as to whether or not angiotensin terminates its own action by stimulating C-kinase. To answer this question we studied the time course of angiotensin-induced activation of phospholipase C by measuring the production of both inositol phosphates and diacylglycerol. When $[^3H]$ inositol-labelled cells were stimulated by 1 nM-angiotensin in the absence of Li^+ , $[^3H]$ inositol trisphosphate increases rapidly to a steady state and remains elevated for at least 25 min. When 100 nM-TPA is added 20 min after the addition of angiotensin, $[^3H]$ inositol trisphosphate decreases gradually (Fig. 5). However, when 5 nM-TPA is added at 20 min, $[^3H]$ inositol trisphosphate does not change significantly (results not shown). In the next set of experiments we measured the time course of changes in diacylglycerol in the angiotensin-treated cells. As Fig. 6 shows, angiotensin causes a rapid increase in diacylglycerol. Diacylglycerol then decreases to a steady-state value that is 140% of the unstimulated value and remains there for at least 30 min. When 100 nM-TPA is added at 20 min, diacylglycerol decreases to a level that is lower than the unstimulated value. By contrast, 5 nM-TPA added at 20 min has no effect on diacylglycerol content (results not shown).

Fig. 5. Time course of angiotensin-induced changes in inositol trisphosphate

$[^3H]$ inositol-labelled cells were stimulated for the indicated time by 1 nM-angiotensin in the absence of Li^+ . In some experiments, 100 nM-TPA was added at 20 min ($\circ$). Values are means $\pm$ S.E.M. for three to four experiments.

Fig. 6. Time course of angiotensin-induced changes in diacylglycerol

Cells were stimulated for the indicated time by 1 nM-angiotensin. In some experiments, 100 nM-TPA was added at 20 min ($\circ$). Diacylglycerol was measured as described in the Materials and methods section and expressed as a percentage of unstimulated control. Values are means $\pm$ S.E.M. for three to four experiments. The diacylglycerol content of unstimulated cells is 18.6 ± 8.2 pmol/ 10^7 cells ($n = 4$).

DISCUSSION

The present results provide additional insights into the role of C-kinase in the adrenal glomerulosa cell. They address three questions: first, does TPA treatment attenuate angiotensin action?; secondly, does TPA exert its action by stimulating C-kinase?; and thirdly, what is the physiological relevance of TPA-induced inhibition of phospholipase C?

Results shown in Figs. 1 and 2 indicate that pretreatment with TPA abolishes angiotensin-induced aldosterone secretion. As Fig. 3 shows, TPA abolishes angiotensin-induced inositol phosphate production. Thus, as in other systems, TPA inhibits the agonist-induced activation of $PtdIns(4,5)P_2$ - and possibly

PtdIns4P-specific phospholipase C. Although the site of TPA action cannot be defined by the results obtained in the present study, TPA inhibits activation of phospholipase C by either blocking the binding of angiotensin to its receptor or affecting the transducing system between receptor and phospholipase C. The latter seems more likely, since TPA-induced inhibition is not overcome by increasing the dose of the agonist.

A question to address is whether or not TPA inhibits agonist action by activating C-kinase. The fact that a synthetic diacylglycerol, but neither inactive phorbol ester nor phorbol, partially reproduces the TPA effect suggests that TPA is acting, at least partly, on C-kinase. We have shown previously that TPA induces a slow, but sustained, increase in aldosterone secretion, and that the maximum effect of TPA is obtained at 5 nM (Kojima *et al.*, 1983). By contrast, more than ten times as much TPA is required to attenuate angiotensin action. At present, the reason for the discrepancy is uncertain. A possible explanation is that the mode of action of TPA may be somewhat different when used at high concentration. In this regard, it is noteworthy that the Ca^{2+} -dependency of TPA action is different when used at high concentration. As reported previously, the stimulatory effect of 5 nM-TPA on aldosterone secretion is dependent on both extracellular and intracellular Ca^{2+} (Kojima *et al.*, 1983), whereas that of 100 nM-TPA is totally independent of Ca^{2+} (I. Kojima & E. Ogata, unpublished work). It is not clear whether a high dose of TPA activates C-kinase *in situ* in a Ca^{2+} -independent manner or whether a high dose of TPA has actions other than stimulating C-kinase. If TPA acts solely on C-kinase, high concentrations of TPA may alter the functional state of C-kinase and the C-kinase will become Ca^{2+} -independent. Further studies are clearly necessary to solve this problem.

Previous reports of TPA-induced inhibition of agonist action have not determined the physiological relevance of the phenomenon. A key question is whether the activation of C-kinase *in situ* terminates agonist action by inhibiting phospholipase C. To clarify this, we chose two approaches. First, we studied the time course of activation of phospholipase C. If activation of C-kinase leads to the inhibition of phospholipase C, it would be expected that agonist-induced hydrolysis of PtdIns (4,5) P_2 would be self-limited. Results obtained do not support this notion (Figs. 5 and 6). When [^3H]inositol-labelled cells are stimulated by angiotensin, the cell [^3H]inositol trisphosphate content remains elevated for at least 25 min, although isomers of inositol trisphosphate were not measured in the present study. Likewise, changes in the cell diacylglycerol content are sustained; after an initial burst, diacylglycerol remains elevated for at least 30 min.

Although the remote possibility that angiotensin inhibits degradation of inositol phosphate and diacylglycerol cannot be totally excluded, the present results support the notion that activation of phospholipase is persistent in this system. Hence, angiotensin-induced activation of C-kinase does not terminate its action. It is, however, possible that sustained activation of phospholipase C is under tonic inhibition via C-kinase. Such an inhibition, if any, may have only a small physiological significance. The second approach was to determine the effect of TPA on activated phospholipase C. When a high, but not a low, dose of TPA was added after 20 min of angiotensin addition, production of inositol trisphosphate

and diacylglycerol is attenuated. This observation indicates that angiotensin and a high dose of TPA have different effects on phospholipase C, even though both activate C-kinase in glomerulosa cells. Thus the inhibitory action of TPA may be a pharmacological effect with little physiological significance, at least in the adrenal glomerulosa cell.

In many systems, agonist-induced breakdown of PtdIns (4,5) P_2 is sustained (Aub & Putney, 1984; Dougherty *et al.*, 1984; Thomas *et al.*, 1984; Akhtar & Abdel-Latif, 1984; Masters *et al.*, 1985; Nabika *et al.*, 1985). Therefore the role of C-kinase as 'a negative-feedback modulator' of agonist action should be evaluated critically. Also, interpretation of experimental data obtained by use of high dose of TPA should be done with considerable caution.

This study was supported by grants from the Ministry of Education, Science and Culture of Japan and the Ministry of Health and Welfare of Japan.

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Received 19 December 1985/26 February 1986; accepted 13 March 1986