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ALDOSTERONE SECRETION: EFFECT OF PHORBOL ESTER AND A23187

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The effects of the divalent ionophore, A23187, the phorbol ester, and/or 12-O-tetradecanoyl-phorbol-13-acetate on aldosterone secretion from adrenal glomerulosa cells were compared to those of angiotensin II (AII). AII causes a prompt and sustained increase in secretion. A23187 causes an initial increase followed by a gradual decline to values less than 25 percent of those seen with AII. TPA causes no initial increase but a slowly progressive rise in secretion rate to a less than maximal value. When TPA and A23187 act together, there is a prompt and sustained increase in aldosterone production rate similar to that seen after AII addition. The effect of TPA is dependent on the free Ca^{2+} concentration of the cell cytosol. These results are interpreted in terms of a model of cell activation in which two branches of the calcium messenger system operate to control respectively the initial and sustained phases of the secretory response. The first phase occurs as a consequence of amplitude modulation of the calmodulin branch of the system by a rise in $[Ca^{2+}]_c$, and the second phase as a consequence of the sensitivity modulation of the C-kinase branch by diacylglycerol.

The discovery of the C-kinase by Nishizuka and coworkers has led to a deeper understanding of how the calcium messenger system operates (1-4). There appear to be two branches by which information flows from cell surface to cell interior when platelets are activated by thrombin (5,6). One is mediated by a rise in the $[Ca^{2+}]$ concentration of the cell cytosol leading to the modulation of the function of calmodulin-dependent reactions; the other by a rise in the diacylglycerol content of the plasma membrane leading to the activation of the C-kinase. Each branch can be activated separately: the $[Ca^{2+}]_c$-calmodulin branch by the divalent ionophore, A23187 (7); and the C-kinase branch by diolein or the phorbol ester, 12-O-tetradecanylphorbol-13-acetate (TPA) (5,6). Activation of either branch alone causes a less than maximal release of serotonin from plate-

Abbreviations: BSA; bovine serum albumin: TPA; 12-tetradecanoyl-phorbol-13-acetate: EGTA; ethylene glycol bis (β-amino-ethyl ester)-N,N,N;,N'-tetraacetic acid: $[Ca^{2+}]_c$; free calcium concentration in the cell cytosol: DG; diacylglycerol: AII; angiotensin II.

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lets. Activation of both branches by the stimultaneous addition of A23187 and TPA induces a maximal rate of serotonin secretion comparable to that seen after thrombin addition. These data led us to consider the possibility that these two branches might have specific temporal roles in the activation of cells, such as those of the adrenal glomerulosa which respond to the sustained presence of an extracellular messenger with a sustained response. Angiotensin II is a major hormonal regulator of aldosterone secretion (8). It produces a prompt and sustained increase in aldosterone secretion that is maintained as long as the angiotensin is present (8). In producing this response, angiotensin appears to act via the calcium messenger system: this hormone is effective only if extracellular calcium is present (9,10); its action is blocked by the calcium channel blockers, Verapamil and D-600 (9-12); it stimulates both the efflux of calcium from an intracellular pool (12,13), and an uptake of calcium into the cell across the plasma membrane (10); and the divalent cation ionophore, A23187, can induce a calcium-dependent increase in aldosterone production (9,10). We examined the effects of A23187 and TPA, alone or in combination, upon aldosterone production from isolated adrenal glomerulosa cells, and compared the time courses of their action with that of angiotensin II.

Materials and Methods

Materials: Angiotensin II, 12-O-tetradecanoyl-phorbol-13 acetate (TPA), 4 - phorbol-12,13-didecanoate, A23187 and collagenase were obtained from Sigma. Sephadex G-15 was obtained from Pharmacia.

Adrenal Glomerulosa Cells

Porcine adrenal glands were obtained in a local slaughterhouse. These were kept in ice-cold Krebs-Ringer-Bicarbonate buffer. After removal of the surrounding adipose tissue, they were cut into two flat pieces. Both medulla and inner zone of the cortex were carefully scraped off with a blade. The thin outer tissue with the capsule was finely minced with scissors and washed several times with calcium free Krebs-Ringer-Bicarbonate buffer with 5.5 mM glucose (KRBG buffer) until the supernatant became clear. The minced fragments were suspended in KRBG buffer containing 0.5 mM calcium, 0.2% bovine serum albumin (BSA) and collagenase (1 mg/ml) equilibrated with 95% O_2, 5% CO_2 gas and incubated for digestion with shaking at 37°C for 60 min. The enzyme-treated tissue was dispersed by repeated aspiration through a polyethylene tube. The dispersed cells were filtered through nylon mesh and washed three times with KRBG buffer containing 0.5 mM calcium and 0.2% BSA.

Perifusion

The perifusion system employed was a modification of the method described elsewhere (13) using 4 flow through chambers in a single lucite block. Each chamber contained a glass wool plug approximately 5 mm high upon which five million cells suspended in Sephadex G-15 were layered. The lucite block was

placed in a water bath at 37°C and each chamber was perfused with KRBG buffer containing 0.5 mM calcium and 0.2% BSA using a multichannel Rainin Miniature Peristaltic Pump. The flow rate was 0.5 ml/min and effluent from each chamber was collected every four min. Aldosterone output was measured by radioimmunoassay using anti-aldosterone antibody from National Hormone and Pituitary Program. In some experiments, the concentration of calcium in the medium was adjusted from 0 to 1.0 μM by using Ca-EGTA buffer (14). TPA and non-active phorbol ester were dissolved in acetone and A23187 was dissolved in ethanol. Final concentration of each solvent was less than 0.1% and neither acetone nor ethanol had any effect on aldosterone production at these concentrations.

Results

Previous work employing a static incubation system had shown that A23187 in the dose range of 1.25 μM (when bovine serum albumin was present) induced an aldosterone secretory response that was 30-40 percent of that induced by maximally stimulating concentrations of angiotensin II (10). Using a static incubation system, adrenal glomerulosa cells were incubated with increasing concentrations of TPA for two hrs. Addition of TPA in a concentration as low as 1.0 nM has a significant effect on aldosterone production, and a concentration of 5.0 nM has a maximal effect. The maximal effect produced by 5 nM TPA is 30-50 percent of that seen after AII (1×10^{-9} M) addition (5.4 ± 0.6 ng/10^6 cells/hr vs 12.5 ± 1.0 mg/10^6 cells/hr).

The secretory responses of isolated adrenal glomerulosa cells perifused with 10^{-9} angiotensin II (AII), 1.25 μM A23187, 5 nM TPA, or a combination of A23187 and TPA are shown as a function of time in Figure 1. When AII is employed, aldosterone secretion begins to increase in about 10 min, rises to a plateau over the next 10 min, and then stays at this plateau value for at least 90 min (Fig. 1A). When 1.25 μM A23187 is added to the perifusate, there is a small immediate increase in aldosterone secretion rate starting at about 10 min which reaches only about 50 percent of the maximal value seen after AII addition. This rate then declines gradually over the next 40-80 min to a rate which is only about 25 percent of that seen after AII addition (Fig. 1B). When 5 nM TPA is added to the perifusate, there is no immediate increase in aldosterone secretory rate, but a slowly developing response (Fig. 1C) which is only 20 percent of that seen after AII addition after 30 min of perifusion, and 50-60 percent after 90 min (data not shown). When both TPA and A23187 are added together, the

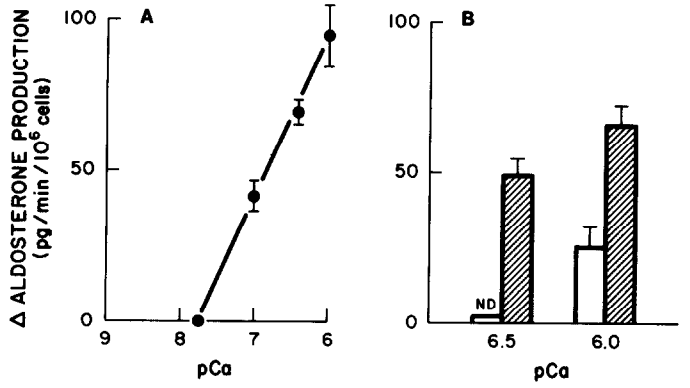

Figure 1. Comparison of time course of aldosterone secretory response to angiotensin II, A23187, TPA, and A23187 + TPA. Cells were perifused with KRBG buffer containing 0.5 mM calcium and 0.2% BSA for 40 min. At time 0, either 10^{-9} M angiotensin II (A), 1.25 μM A23187 (B), 5 nM TPA (C) or 1.25 μM A23187 + 5 nM TPA (D) was added to perifusate. Aldosterone output is expressed as absolute rates of production and each point is the mean value ± S.D. obtained from three separate experiments run on three different cell preparations.

temporal pattern of the aodosterone secretory response (Fig. 1D) is similar to that seen after addition of AII (Fig. 1A).

To provide evidence that TPA acts via the C-kinase pathway, several experiments were done. First, glomerulosa cells were perifused with 4 -phorbol-12,13-didecanoate, a non-active phorbol ester (1). This phorbol ester has no effect, alone or in combination with A23187. Second, cells were incubated with high concentrations of the ionophore (5 μM) to make the plasma membrane freely permeable to calcium ion and effectively clamp the intracellular free calcium concentration, and then perifused with media containing varying low concentrations of free Ca^{2+} (fixed by Ca-EGTA buffers and no BSA). Under these conditions, the action of TPA is dependent upon the intracellular calcium concentration as shown in Figure 2A. The data show that the effect of TPA is directly dependent on intracellular Ca^{2+} concentration.

A second prediction was that at a fixed intracellular free Ca^{2+}, angiotensin would stimulate aldosterone secretion even though it did not change the Ca^{2+} concentration because it would stimulate the C-kinase reaction. Experiments to test this prediction were carried out by perifusing porcine cells in medium containing Ca^{2+}-EGTA buffers with either 0.5 or 1.0 μM free Ca^{2+} concentrations and 5 μM ionophore. The cells were then perifused with either 10^{-9} M AII or

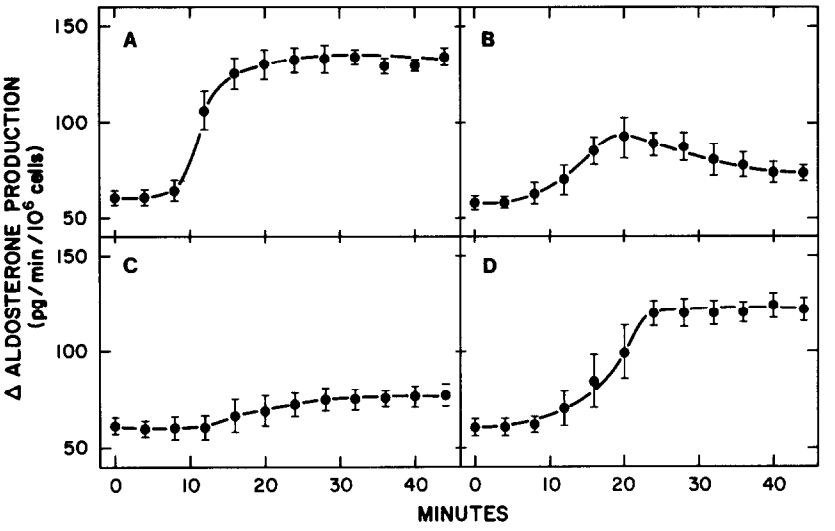

Figure 2. A. The change in rate of aldosterone production as a function of the free Ca^{2+} concentration (plotted as pCa) in porcine adrenal glomerulosa cells incubated with 5 μM A23187 and calcium-EGTA buffers of the appropriate free Ca^{2+} concentration, and then treated for 30 minutes with 5 nM TPA. Aldosterone production is expressed as change from basal rate. The basal rate was zero when the Ca^{2+} concentration was either 0.02, 0.1, or 0.5 μM, and 25 pg/min/10^6 cells when the Ca^{2+} concentration was 1.0 μM. The mean ±S.D. for three separate experiments are plotted.

B. The effect of angiotensin II on aldosterone production in porcine adrenal glomerulosa cells incubated with 5 μM A23187 and a calcium-EGTA buffer having a free calcium concentration of either 0.5 or 1.0 μM. The rates of aldosterone production 40 minutes after addition of either diluent (open bars) or 10^{-9} M AII (dashed bars) for each free Ca^{2+} concentration are shown as mean values ±S.D. for three separate experiments.

diluent. The rates of aldosterone production 45 min after AII addition are shown in Figure 2B. When the free calcium is 0.5 μM (pCa-6.5) the basal rate of aldosterone production is unmeasureable, but addition of 10^{-9} AII causes a significant stimulation. When the free calcium is 1.0 μM (pCa-6.0), the basal rate is approximately 25 pg/min/10^{-6} cells, and addition of AII increases this to 67 pg/min/10^6 cells.

Discussion

The present results extend the observation of Nishizuka and collaborators (1-6) showing that the combination of A23187 and TPA will mimic the action of a natural agonist. Neither agent alone is completely effective in doing so. Hence, the data argue that the C-kinase as well as the calmodulin branch plays a role in the regulation of steroid hormone secretion (Fig. 1,2). An immediate question

is the specificity of TPA action. Castagna et al. (15) showed that TPA acts as a positive sensitivity modulator of C-kinase function. Since then, it has been found that the receptor for TPA in the cell and the C-kinase co-purify (16). These results have led to the conclusion that a major, if not sole, cellular site of action of TPA is on the C-kinase. This conclusion is supported indirectly in the present study by the demonstration that the effect of TPA in the glomerulosa cell is dependent on the intracellular calcium concentration (Fig. 2A), a result to be expected if TPA acts via the C-kinase pathway. Furthermore, the fact that angiotensin II stimulates aldosterone production even when cytosolic Ca^{2+} concentration is fixed by ionophore treatment and Ca-EGTA buffers (Fig. 2B) supports the hypothesis that angiotensin II stimulates the production of a second messenger other than Ca^{2+}, presumably diacylglycerol, an endogenous ligand for the TPA receptor.

If one considers these data in the context of our present knowledge of the function of the calcium messenger system (7-20), it is possible to present a pausible model of events in this system during the process of cell activation. When an extracellular messenger binds to its surface receptor, three interrelated events occur: a) release of Ca^{2+} from an intracellular pool(s); b) an increase in the Ca^{2+} permeability of the plasma membrane; and c) an increase in the DG content of the plasma membrane (4). The first two lead to a transient increase in $[Ca^{2+}]_C$ (7,21-25). The rise in $[Ca^{2+}]_C$ is detected by calcium receptor proteins such as calmodulin, and as a consequence a number of calmodulin-regulated enzymes (and other calcium-dependent processes) are activated leading to an initial cellular response. One of these calmodulin-regulated response elements is the plasma membrane calcium pump (14,26,27). Its activation, along with the uptake of Ca^{2+} from cytosol into mitochondria (19,20), cause a fall of $[Ca^{2+}]_C$ to lower values (7,21-25). These values are probably less than 0.5 μM. The simultaneous rise in both the $[Ca^{2+}]_C$ and the DG content of the plasma membrane leads to the activation of C-kinase. This enzyme catalyzes the phosphorylation of a subset of proteins different from the subset which serve as substrates for the calmodulin-dependent kinase (1-6). These proteins are important in bringing

about and maintain long term response of the cell. The unique feature of this C-kinase pathway is that DG is a positive sensitivity modulator (18) of this calcium-activated enzyme (2). An increase in its concentration increases dramatically the affinity of Ca^{2+} for the enzyme and increases the V_{max} of the enzyme when activated by Ca^{2+}. This model proposes that the C-kinase pathway provides a type of gain control in the calcium messenger system allowing for nearly maximal rates of cellular output at less than maximal levels of calcium messenger intput.

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