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POSSIBLE ROLE OF CALCIUM UPTAKE AND CALMODULIN IN ADRENAL GLOMERULOSA CELLS: EFFECTS OF VERAPAMIL AND TRIFLUOPERAZINE

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Abstract—The effects of verapamil and trifluoperazine were examined on isolated rat adrenal glomerulosa cells so as to assess the role of calcium ion influx and calmodulin in the function of this cell population. Verapamil (10^{-5} and 10^{-4} moles/l) slightly reduced the basal production rate of aldosterone and strongly inhibited the response to angiotensin II, potassium ions, corticotrophin (ACTH) and dibutyryl cyclic AMP (db-cAMP). The concentration of verapamil required to reduce the response to these agonists by 50% varied between 2 and 6 μmoles/l. Trifluoperazine (30 μmoles/l) slightly increased the basal production rate of aldosterone. The response to angiotensin and potassium was variably antagonized by 3 μmoles/l trifluoperazine and completely inhibited by the drug at 30 μmoles/l. The antagonist at a concentration of 3 μmoles/l exerted either a facilitatory or inhibitory effect on the response to ACTH and db-cAMP, depending on the concentration of the agonist. Trifluoperazine at a concentration of 30 μmoles/l reduced the response to both agonists to a level which was 2–3 fold higher than that observed in appropriate control samples. The present results indicate that (1) calcium influx is an essential event in the aldosterone stimulating action of angiotensin II, potassium ions, ACTH and cyclic AMP; (2) stimulation by angiotensin II and potassium ions are completely dependent on calmodulin; (3) stimulation by ACTH and cyclic AMP is mediated by calmodulin-dependent and independent mechanisms.

The role of the calcium ion in steroidogenesis was revealed as early as 1953 by Birmingham and coworkers [1]. Although the mode of action of this ion has not yet been elucidated, it is generally accepted that calcium may exert an effect at several sites in the control of steroid production (for review see [2, 3]). The calcium-dependence of the stimulation of aldosterone production by angiotensin II [4–9], potassium [4, 7, 8] and corticotrophin (ACTH) [5, 7, 8, 10] has been amply verified. Considering that ACTH stimulates the production of aldosterone via cyclic AMP [9–11] while the action of angiotensin II and potassium appears to be independent of the cyclic nucleotide [6, 8, 9, 12, 13], a messenger role of calcium ions in glomerulosa cells deserves special attention. Available reports [13, 14] on radiocalcium fluxes in isolated glomerulosa cells have not been conclusive regarding the role of enhanced calcium influx during stimulation. Since the effect of extracellular calcium ions may be due to a specific calcium uptake with respect to both quantity and localization [15], the application of inhibitors of calcium influx may provide valuable information. Therefore, we have repeated and completed previous studies [6, 10, 16] on the effect of verapamil, an inhibitor of calcium uptake (cf. [17]). In subsequent experiments we examined the possible role of calmodulin, a calcium-dependent regulator of many enzymes (for review see [18, 19]). This problem was approached by the application of the phenothiazine tranquillizer drug trifluoperazine, an inhibitor of calmodulin (cf. [18, 20]).

MATERIALS AND METHODS

Materials. Materials used for cell isolation and incubation as well as in aldosterone analysis have been described [21].

Angiotensin II (asp^1-val^5-angiotensin II-β-amide, Hypertensin) and 1-24 ACTH (Synacthen) were obtained from Ciba-Geigy (Basle, Switzerland), dibutyryl cyclic AMP (db-cAMP) from Calbiochem (Loewengraben, Switzerland), verapamil from Knoll (Liestal, Switzerland), and trifluoperazine from Smith Kline & French Laboratories (Philadelphia, PA).

Cell isolation and incubation. Glomerulosa cell suspensions were prepared from the adrenal capsular tissue of male Sprague–Dawley (CFY) rats after treatment with collagenase, as detailed by Enyedi and Spät [21]. The yield was 130,000 glomerulosa cells per rat on average ($n = 18$).

The cells (about 65,000 per sample) were incubated in Teflon vials at 37° for 90 min in an atmosphere of 95% O_2 and 5% CO_2. The incubation was carried out in 1 ml mixture of Medium 199 (Wellcome, Beckenham, Kent, U.K.) and Krebs–Ringer bicarbonate–glucose solution (1:2, v/v) containing 2 g human serum albumin (fraction V, Humán, Budapest, Hungary) per litre. Potassium concentration was 3.6 mmoles/l unless otherwise indicated. Each incubation was carried out in duplicate. Basal aldosterone production was 2.11 ± 0.39 (S.E.M.)

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pmoles/adrenal per 90 min as an average of 18 experiments reported in this paper.

Estimation of aldosterone. Aldosterone content of the cell suspension was estimated by radioimmunoassay as previously described [21].

Statistical analysis. Given the considerable variations in the production rate of aldosterone between separate experiments, the values were expressed as the ratio of the production rate of the stimulated sample (with or without inhibitor) and that of the matched, non-stimulated control sample (without any inhibitor). Means ± S.E.M. are given. Individual values in the figures represent the mean of duplicate incubations. Significance of differences was estimated by paired-sample *t* test.

RESULTS

Effect of verapamil. The effect of verapamil *per se* was examined in the concentration range of 10^{-6} to 10^{-4} moles/l. The drug had a slight inhibitory action on the basal production rate of aldosterone. At a concentration of 10^{-5} and 10^{-4} moles/l the production rate of the hormone decreased by 20 per cent on average (Fig. 1).

The response to angiotensin II added at maximally effective concentration (25 nmoles/l) was reduced by verapamil in a concentration-dependent manner. The concentration required to inhibit the response of aldosterone production by 50% (IC_{50}) was 2.3 ± 0.4 μmoles/l. ACTH was added to the cells at a concentration of 10 nmol/l, which elicited half-maximal response. The inhibitory effect of verapamil on this response was similar to its effect on angiotensin-stimulated cells, IC_{50} was 2.4 ± 0.1 μmoles/l. The response to db-cAMP (100 μmoles/l) was also inhibited by verapamil, IC_{50} was 5.4 ± 0.4 μmoles/l. When the cells were stimulated with potassium ions (5.7 mmoles/l), 3 μmoles/l verapamil reduced the rate of aldosterone production: the 4.81 ± 0.20-fold stimulation was reduced to a 2.91 ± 0.18-fold one ($n = 3$) which is approximately a 50% decrease of the response.

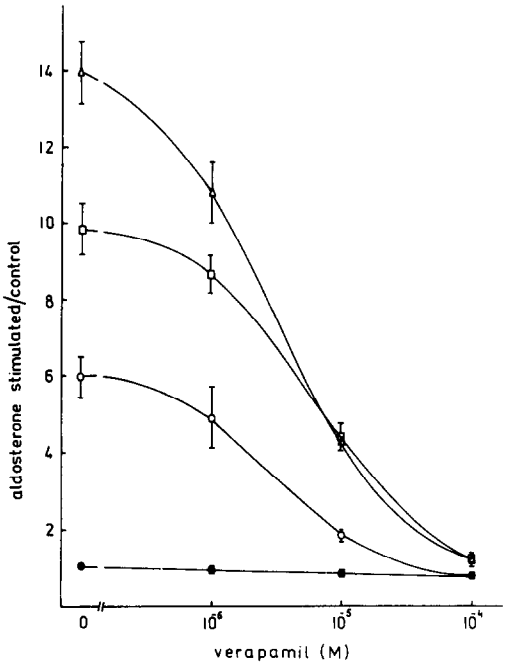

Fig. 1. Effect of verapamil on the production rate of aldosterone by isolated glomerulosa cells. Stimulation ratios of aldosterone were expressed by dividing the production rate of the experimental sample with that of the non-stimulated, non-inhibited control sample. The cells were unstimulated (—●—) or stimulated with 25 nmol/l angiotensin II (—○—), 10 μmoles/l dibutyryl cyclic AMP (—□—) or 10 nmoles/l corticotrophin (—△—), respectively. (Means ± S.E.M., $n = 3$ duplicates, the lines are drawn by eye.)

Effect of trifluoperazine. Trifluoperazine added to the incubation medium at 3 μmoles/l, had no effect

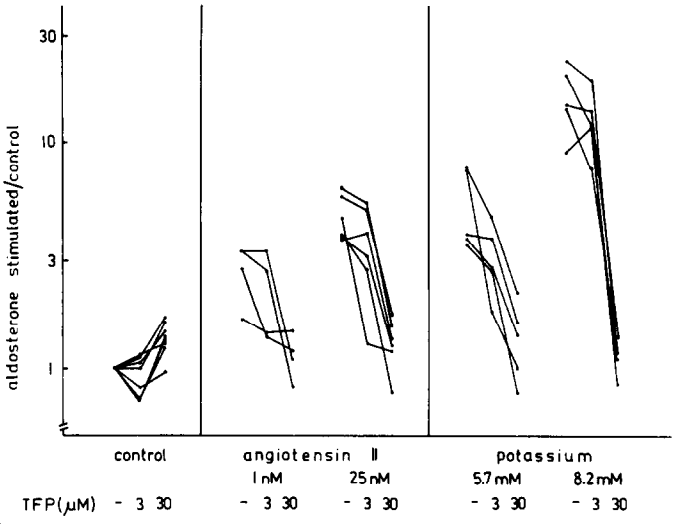

Fig. 2. Effect of trifluoperazine (TFP) on the production of aldosterone by control cells or by cells stimulated with angiotensin II (1 or 25 nmoles/l) or potassium (5.7 or 8.2 mmoles/l). The production rate of aldosterone (ordinate) has been expressed as described in the legend to Fig. 1. Each point represents the mean of duplicate determinations. Solid lines connect data corresponding to samples of the same batch of cells. (Note the logarithmic scale of ordinate!)

on the basal production rate of aldosterone; at 30 μmoles/l it evoked a 36 ± 6% increase ($P < 0.001$, Fig. 2).

At a concentration of 3 μmoles/l the drug inhibited the response to angiotensin II (1 and 25 nmoles/l) to a variable extent while 30 μmoles/l of it reduced the production rate to the level observed with trifluoperazine-treated controls (Fig. 2).

Trifluoperazine exerted a similar effect on potassium-stimulated cells. The response to potassium (5.7 and 8.2 mmoles/l) was variably antagonized by the inhibitor at a concentration of 3 μM while at the higher concentration the drug completely blocked the stimulatory action of potassium (Fig. 2).

The effect of trifluoperazine on the response to ACTH (3–300 nmoles/l) and db-cAMP (25–1000 μmoles/l) (Fig. 3) was remarkably different from that observed in angiotensin- or potassium-stimulated cells. At a concentration of 3 μmoles/l the drug reduced the response to 3 nmoles/l ACTH and 25 μmoles/l db-cAMP; it slightly but consistently potentiated the response to 10 nmoles/l ACTH and 100 μmoles/l db-cAMP and had no consistent effect on the stimulatory action of 300 nmoles/l ACTH and 1 mmole/l db-cAMP. The response to both agents was seriously reduced by the inhibitor added at a concentration of 30 μmoles/l but the inhibition was still not complete. The production rate was maintained in each group at a statistically significantly higher level than that observed in trifluoperazine-treated controls (control vs 10 nmoles/l ACTH or 25 μmoles/l db-cAMP: $P < 0.005$; control vs 300 nmoles/l ACTH, 100 μmoles/l or 1000 μmoles/l db-cAMP: $P < 0.05$).

DISCUSSION

In the first series of the present experiments we have examined the effect of verapamil on the function of rat glomerulosa cells. The drug slightly depressed the basal production of aldosterone and significantly reduced the response of the cells to physiological stimuli such as angiotensin II, potassium and ACTH. This observation gives support to the contention that calcium influx plays an essential role in the stimulation of aldosterone production.

D-600, a compound related to verapamil, inhibited angiotensin- and potassium-induced aldosterone production by isolated rabbit glomerulosa cells at very high concentration (10^{-4} mole/l) only [7]. On the other hand, verapamil inhibited the stimulation of rat glomerulosa tissue [6, 10] or cells [16] by physiological stimuli. Fakunding *et al.* [16] reported that the IC_{50} of verapamil in angiotensin- or potassium-stimulated cells was an order of magnitude lower than that in cells stimulated with ACTH. In the present experiments IC_{50} for verapamil was comparable with the three agonists examined.

Omission of calcium from the incubation medium [22–24] as well as addition of verapamil or D-600 [15, 25] have been repeatedly found to diminish, although not abolish the stimulatory effect of cyclic AMP on glucocorticoid production. Similarly, the stimulatory effect of cyclic AMP on aldosterone production was reduced in a calcium-free medium [8]. In the present experiments verapamil exerted a concentration-dependent inhibition of db-cAMP-induced aldosterone-production. (This observation, of course, does not exclude an additional effect of verapamil on the formation of cyclic AMP, as found by Fakunding *et al.* [16] and Podesta *et al.* [15].) The sensitivity to verapamil of the action of cyclic AMP suggests that increased formation of the nucleotide may result in the opening of calcium channels in the plasma membrane, as has been previously described, e.g., in the myocardium [26, 27], in skeletal muscle [28] or in presynaptic membranes [29].

In view of the possible enhancement of calcium influx into glomerulosa cells after stimulation with physiological stimuli the mode of action of calcium

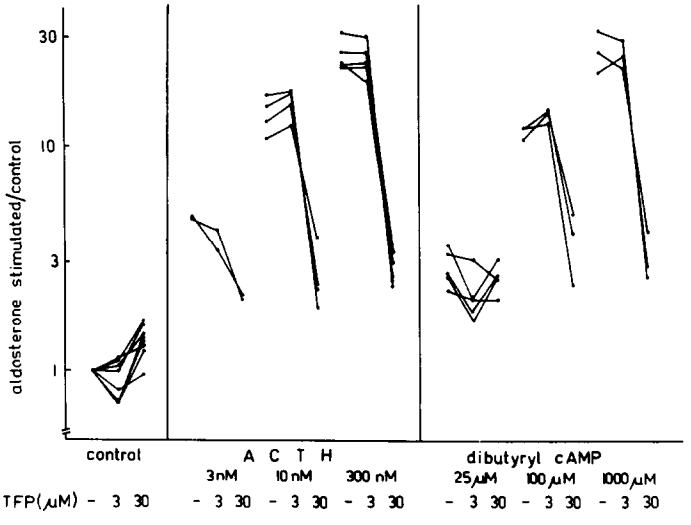

Fig. 3. Effect of trifluoperazine (TFP) on the production of aldosterone by control cells or by cells stimulated with corticotrophin (ACTH; 3, 10 or 300 nmoles/l) or dibutyryl cyclic AMP (25, 100 or 1000 μmoles/l). The production rate of aldosterone (ordinate) has been expressed as described in the legend to Fig. 1. Each point represents the mean of duplicate determinations. Solid lines connect data obtained with samples of the same batch of cells. (Note the logarithmic scale of ordinate!)

ions has to be cleared. One purpose of the present study was to assess whether the action of calcium ions is mediated by calmodulin. Calmodulin has been recently detected in the adrenal cortex by means of an immunofluorescent technique by Harper *et al.* [30]. Since no special mention was made of the zone glomerulosa in this paper the subject of their study was probably the wide deeper zone (zona fasciculata and reticularis). In the present work we examined the effect of trifluoperazine, a known inhibitor of calmodulin. When aldosterone production was stimulated by angiotensin II or potassium, trifluoperazine at low concentration (3 μmoles/l) evoked a partial, and at a higher concentration (30 μmoles/l) a complete, inhibition of the response. The response to ACTH and b-cAMP was slightly and variably affected by trifluoperazine added at low concentration (3 μmoles/l). At the higher concentration (30 μmoles/l) the drug reduced the ACTH- and db-cAMP-induced aldosterone production to a rate about twice as high as shown by trifluoperazine-treated control cells. Since the rate of residual production of aldosterone in the presence of 30 μmoles/l trifluoperazine did not depend on the degree of stimulation by the various concentrations of ACTH or db-cAMP, it may be presumed that—in contrast to the effect of angiotensin II and potassium ions—the effect of ACTH and its second messenger, cyclic AMP is mediated only partly by calmodulin-dependent pathways. The trifluoperazine-sensitive pathway seems to be activated by higher concentrations of cyclic AMP and to have a higher capacity than the insensitive pathway.

In view of the complete inhibition by 100 μmoles/l verapamil and incomplete inhibition by 30 μmoles/l trifluoperazine of the effect of ACTH and db-cAMP, it may be postulated that there is a step(s) in the action of cyclic AMP which requires calcium ions but is not less dependent on calmodulin (cf. [31]).

Elevation of the concentration of cytosolic calcium ions is generally followed by increased formation of cyclic GMP (cf. [32]). Cyclic GMP is a weak stimulator of corticosteroid production, further the production of cyclic GMP in glomerulosa cells does not change in response to angiotensin II or potassium [12]. Therefore, the involvement of cyclic GMP in the phenomena described above seems to be unfounded.

Trifluoperazine, due to its lipophilic character, may induce alterations in cell functions without inhibiting calmodulin. Although such effects may be expected at concentrations higher than those applied by us [33], inhibition of calmodulin-dependent activity by trifluoperazine is only one of the criteria proposed by Cheung [34] for calmodulin-regulated reactions. Further studies are required to provide direct evidence for the role of calmodulin in glomerulosa cells and to demonstrate its precise site of action in the control of aldosterone synthesis.

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