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THE EFFECT OF ANGIOTENSIN II ON ARACHIDONATE METABOLISM IN ADRENAL GLOMERULOSA CELLS

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Abstract—The effect of angiotensin II on arachidonate metabolism was examined in rat adrenal glomerulosa cells. Incorporation of both [^{3}H]arachidonate and [^{32}P]phosphate into phosphatidylinositol (PI) were significantly stimulated by angiotensin II. These effects were abolished by lithium, a cation, which was found suitable to prevent increased synthesis of PI in our previous study (T. Balla *et al.*, *FEBS Letters* **171**, 179, 1984). On the other hand, the phospholipase A_2 inhibitor mepacrine failed to inhibit the increased labelling of PI. These observations suggest that the increased ^{3}H labelling of PI occurs via CDP-diacylglycerol, and not via enhanced deacylation–reacylation cycle. The validity of this assumption was further supported, since angiotensin II failed to stimulate the formation of lyso-PI, as examined by both [^{32}P]phosphate incorporation and pulse-chase techniques.

Angiotensin II decreased the incorporation of [^{3}H]arachidonate into phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Considering that we did not find arachidonate release either from phospholipids or from other possible arachidonate sources this decrease may not be due to dilution of the tracer. Thus we assume that angiotensin II may induce a shift in phospholipid synthesis from PC and PE to phosphoinositides.

These observations indicate that the enhanced hydrolysis and synthesis of PI in response to angiotensin II is not associated with increased phospholipase A_2 activity in adrenal glomerulosa cells.

Stimulation of phospholipase A_2 (EC 3.1.1.4) activity, and the consequent arachidonic acid release has been found to play an important role in stimulus-secretion coupling in several tissues (cf. [1, 2]). Moreover, one of the main sources of released arachidonate is phosphatidylinositol (PI),* a lipid which shows characteristic changes in response to Ca^{2+}-mobilizing hormones [3].

In glomerulosa cells the turnover of PI is stimulated by the Ca^{2+}-mobilizing peptide angiotensin II, while the cyclic AMP-mediated ACTH has no such effect [4, 5]. The relative role of phospholipase C (EC 3.1.4.10), splitting phosphoinositides into DAG and inositol-phosphates and that of phospholipase A_2, producing lyso-PI and free fatty acids, in the breakdown of PI has not yet been elucidated in the zona glomerulosa. The purpose of the present study was to examine the relation of arachidonate turnover to phospholipid metabolism and the possible contribution of phospholipase A_2 activation to the early effects of angiotensin II in adrenal glomerulosa cells.

MATERIALS AND METHODS

Materials used for cell isolation and incubation have been described [6]. Glomerulosa cells were prepared from the adrenal capsular tissue of male Sprague–Dawley (CFY) rats (body weight, 250–380 g) by means of digestion with collagenase. A previously described method [6] was applied, with the modification that bicarbonate in the Krebs–Ringer [7] solution was replaced by Hepes (final concentration, 20 mM, pH 7.4) and, accordingly, incubations were carried out under ambient air.

Pooled cells equivalent to 0.7–1 pair of adrenals were incubated in 100 μl of a mixture of modified Krebs–Ringer–glucose solution and Medium 199 (Wellcome, Beckenham, Kent, U.K.) (2:1, v/v) containing 2 g/l human serum albumin (fraction V) and buffered with Hepes (20 mM, pH 7.4). Concentration of potassium and calcium was 3.6 mM and 1.8 mM, respectively. The incubations were carried out in Eppendorf centrifuge tubes at 37°. Sodium ortho[^{32}P]phosphate (0.5–1.7 GBq/l) and/or [^{3}H]-arachidonic acid (70–120 MBq/l, 7 TBq/mmole) (both obtained from the Isotope Institute of the Hungarian Academy of Sciences, Budapest) and asp^1-ileu^5-angiotensin II (Serva, Heidelberg, F.R.G.) or corticotropin (1-24 ACTH, Synachten, Ciba-Geigy, Basel, Switzerland) were added simultaneously, unless otherwise stated. Lithium chloride and mepacrine (quinacrine, Sigma Chemical Co., St. Louis, MO) were added 30 min before the label and angiotensin.

When breakdown of phospholipids was studied, the cells were incubated in the presence of radiophosphate for 1 hr, washed and chased for 3 hr in a medium without radioactive phosphate. Angiotensin or solvent was added in the 180th min of this second incubation. When breakdown of arachidonate-labelled lipids was studied, the cells were incubated in the presence of [^{3}H]arachidonate for 4 hr, washed twice and resuspended in arachidonate-free medium. In both types of breakdown experiments (^{32}P or ^{3}H)

* Abbreviations: PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DAG, diacylglycerol; ACTH, adrenocorticotropin; Hepes, 4-/2-hydroxyethyl/-1-piperazine-ethanesulphonic acid.

the cell content in the final suspension corresponded to about one pair of adrenals per sample.

The following extraction methods were applied: for major phospholipids in kinetic studies (Fig. 2) and inhibitor studies (Fig. 3), Farese *et al.* [8]; for PI and lyso-PI in [^{32}P]phosphate incorporation experiments (Fig. 4), Creba *et al.* [9]; for phospholipids including lyso-phospholipids (Fig. 5), Billah *et al.* [10]; for major lipid classes (Fig. 6), Folch *et al.* [11].

Lipid separation of the dry extract was achieved by thin layer chromatography on silica gel 60 plates (Merck, Darmstadt, F.R.G.) as follows: for major phospholipids (Figs 2 and 3), Weiss *et al.* [12]; for PI and lyso-PI (Fig. 4), Enyedi *et al.* [13]; for phospholipids including lyso-phospholipids: the first development (chloroform–methanol–2 M ammonia, 7:7:2, v/v) was followed with a system chloroform–methanol–acetic acid–water (50:30:8:2) in the second direction (Fig. 1). For this two dimensional system the plates were preimpregnated with 75 mM oxalate + 2 mM EDTA + 0.5 M boric acid. Major lipid classes (Fig. 6) were separated with system *n*-hexane–diethylether–formic acid (80:20:2) [14].

Lipids on the plates were visualized by exposing the plates to iodine vapour and/or by autoradiography. Each spot was scraped off and their radioactivity was determined by means of a Beckman LS-250 liquid scintillation counter.

Phospholipids extracted from [^{32}P]phosphate-loaded adrenal fasciculata cells were used as internal standard in the analysis of samples of kinetic studies (Fig. 1).

Means ± S.E.M. are given. The number of observations indicates the mean of duplicate incubations from N separate experiments. The significance of the effect of angiotensin was estimated by means of paired sample *t*-test or three-way analysis of variance [15].

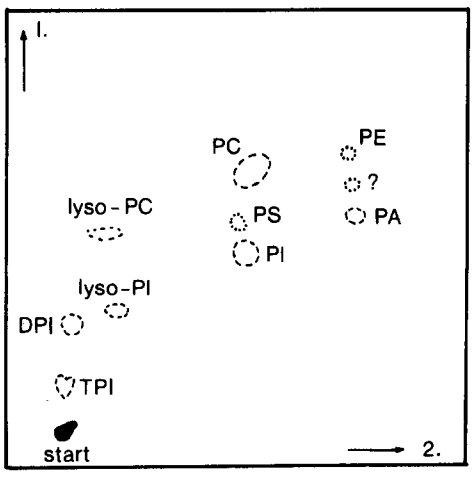

Fig. 1. Separation of phospholipids by two-dimensional thin layer chromatography. Silica gel 60 plates (10 × 10 cm) were preimpregnated with 75 mM oxalate + 2 mM EDTA + 0.5 M boric acid. The first development was performed in chloroform/methanol/2 M ammonia (7:7:2, v/v). After drying the plates, solvent system chloroform/methanol/acetic acid/water (50:30:8:2) was used in the second direction. Abbreviations: TPI, phosphatidylinositol-4,5 bisphosphate; DPI, phosphatidylinositol-4-phosphate; PI, phosphatidylinositol; PC, phosphatidylcholine; PS, phosphatidylserine; PA, phosphatidic acid; PE, phosphatidylethanolamine. Radioactive spots were identified by autoradiography.

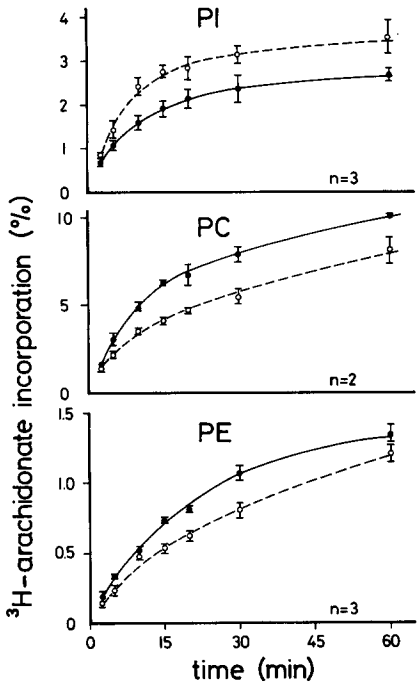

Fig. 2. Incorporation of [^{3}H]arachidonic acid by isolated glomerulosa cells into phosphatidylinositol (PI), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) as a function of time. Cells were incubated at 37° in 100 µl of a mixture of Krebs–Ringer/Hepes/glucose/albumin solution and medium 199 (2:1, v/v) for the times indicated. [^{3}H]Arachidonic acid (7 kBq, 7 TBq/mmole) and angiotensin II (25 nM) (--○--) or solvent (—●—) was added at 0 min. Means ± S.E.M. of three separate experiments are shown, each carried out in duplicates. In one of the three experiments the PC fraction was lost during analysis.

RESULTS

Incorporation of [^{3}H]*arachidonate into phospholipid fractions*

All the three major phospholipid fractions examined incorporated [^{3}H]arachidonate. Angiotensin II, added in a concentration of 25 nM, increased the incorporation into PI ($P < 0.005$, Fig. 2). In contrast to PI, there was a decrease in arachidonate incorporation into PE ($P < 0.005$) and PC (Fig. 2). The stimulatory effect on PI labelling was characteristic of angiotensin since ACTH (3×10^{-7} M) evoked a slight but statistically significant decrease in the incorporation rate in all three phospholipids (data not shown).

Effect of lithium and mepacrine on [^{3}H]*arachidonate and* [^{32}P]*phosphate incorporation into PI and PC*

In order to test whether angiotensin-enhanced incorporation of labelled arachidonate into PI was a phenomenon merely associated to the enhanced synthesis of PI or to a phospholipase A_2-activated

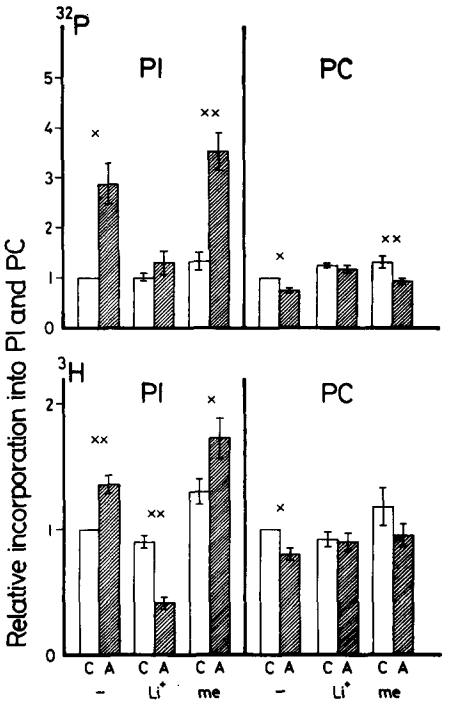

Fig. 3. Incorporation of [^{32}P]phosphate (upper panel) and [^{3}H]arachidonic acid (lower panel) by isolated glomerulosa cells into phosphatidylinositol (PI) and phosphatidylcholine (PC). Isolated glomerulosa cells were incubated in 100 μl incubating medium (for composition see legend to Fig. 2) in the presence of LiCl (10 mM) (Li^+) or mepacrine (100 μM) (me) or without any of them for 30 min. At this time [^{3}H]-arachidonic acid (7 kBq, 7 TBq/mmole) and [^{32}P]phosphate (170 kBq) was added together with angiotensin II (25 nM) (A) (stripped bars) or solvent (open bars) (C). The incubations were carried out for a further 30 min. Radioactivity of phospholipids were related to the non treated control. Means ± S.E.M. of five (PI) or four (PC) experiments are given, each carried out in duplicate. The effect of angiotensin II was compared to the respective, inhibitor-treated control (*$P < 0.05$; **$P < 0.01$).

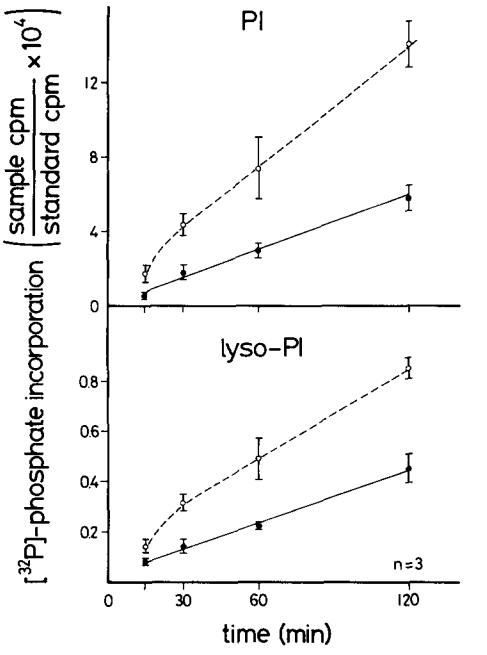

Fig. 4. Incorporation of [^{32}P]phosphate by glomerulosa cells into phosphatidylinositol (PI) and its lyso derivative (lyso-PI) as a function of time. Cells were incubated in 100 μl incubating medium (for composition see legend to Fig. 2) which contained [^{32}P]phosphate (50–100 KBq) at 37°. Angiotensin II (25 nM) (--○--) or solvent (—●—) was added at 0 min. ^{32}P incorporation into phospholipids was expressed as a fraction of the total [^{32}P]phosphate activity of the sample (=standard cpm). Means ± S.E.M. of three separate experiments are shown, each carried out in duplicates.

deacylation–reacylation cycle, the effect of angiotensin was examined in the presence of two inhibitors. Lithium was applied to interrupt the accelerated PI-cycle. It inhibits myo-inositol-1-phosphatase [16–18] and, as demonstrated previously in our laboratory, it completely prevents the agonist stimulated synthesis of PI from inositol and CDP-diacylglycerol [19]. The other drug mepacrine is generally applied to inhibit phospholipase A_2 [20, 21]. In accordance with our previous results [4], stimulation with angiotensin II for 60 min resulted in a threefold increase in the labelling of PI with radiophosphate ($P < 0.05$, Fig. 3). Lithium (10^{-2} M) almost completely abolished this effect. Mepacrine (10^{-4} M) moderately increased the basal incorporation but the stimulatory effect of angiotensin II was preserved ($P < 0.01$, Fig. 3).

Incorporation of [^{3}H]arachidonate into PI was enhanced by angiotensin ($P < 0.01$). This effect could also be prevented by lithium; moreover, angiotensin II decreased the [^{3}H]arachidonic acid incorporation into PI in the presence of the cation ($P < 0.01$, Fig. 3). The effect of mepacrine on ^{3}H labelling did not differ from that on [^{32}P]phosphate incorporation (Fig. 3).

These data obtained with the use of inhibitors suggest that increased incorporation of [^{3}H]arachidonate into PI is a phenomenon associated with increased PI synthesis, and not with an accelerated deacylation–reacylation cycle. Incorporation of [^{32}P]-phosphate as well as [^{3}H]arachidonate into phosphatidylcholine was decreased by angiotensin II ($P < 0.05$). This effect was abolished by lithium but was not influenced by mepacrine (Fig. 3).

Effect of angiotensin II on [^{32}P]phosphate turnover in lyso-PI

A possible activation of phospholipase A_2 by angiotensin was further studied. First we examined the incorporation of radiophosphate into PI and lyso-PI. If angiotensin activates phospholipase A_2 (without increasing the activity of the corresponding acyltransferase), it should increase [^{32}P] incorporation into lyso-PI in a larger extent than into PI. As shown in Fig. 4, angiotensin increased the incorporation into PI and lyso-PI in a comparable manner. This suggests that the increased labelling of lyso-PI only reflects the increase in the specific activity of PI.

In order to eliminate angiotensin-induced elevation of the specific activity of PI, its effect on lyso-

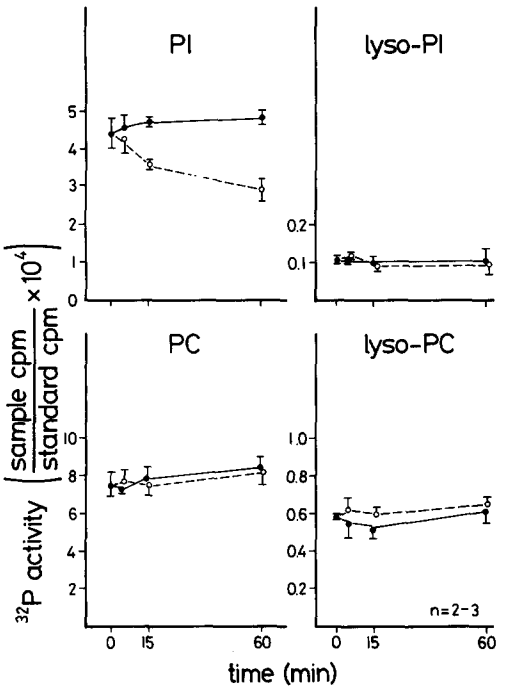

Fig. 5. Radioactivity of phosphatidylinositol (PI), phosphatidylcholine (PC) and their lyso derivatives in isolated glomerulosa cells prelabelled with [^{32}P]phosphate. The cells were incubated in the presence of [^{32}P]phosphate (1.5 GBq/l) at 37° for 1 hr, washed and incubated for a further 3 hr without radioactive phosphate. At this time (taken as 0 min on the figure) angiotensin II (25 nM) (---○---) or solvent (—●—) was added. ^{32}P incorporation into phospholipids was expressed as a fraction of the total ^{32}P-phosphate activity of the sample (=standard cpm.). Means ± S.E.M. obtained from three separate experiments are given, each carried out in duplicates (at t = 60 min, N = 2).

PI production was further examined under pulse-chase conditions. While we found enhanced breakdown of PI throughout the whole observation period lasting from 5 to 60 min, we could not detect any increase in the amount of labelled lyso-PI or lyso-PC (Fig. 5). This result is further evidence against a significant activation of phospholipase A_2.

Effect of angiotensin II on the [^{3}H]*arachidonate content of various lipid classes*

The decreased labelling of PC and PE after stimulation with angiotensin II (Fig. 2) might be attributed to release of unlabelled arachidonic acid from other lipid fractions. To examine such a possibility, [^{3}H]-content of possible arachidonic acid sources, other than phospholipids were examined under pulse-chase conditions. We could not detect changes, however, in ^{3}H-activity either in cholesterol ester or in triacylglycerol fractions after adding angiotensin for 15 or 60 min (Fig. 6). Angiotensin also failed to influence arachidonate release during the incubation period examined (Fig. 6), indicating that there is no arachidonate release from other possible sources.

DISCUSSION

Arachidonate, directly or via the produced prostaglandins or leukotrienes, may modulate stimulus–response coupling [22–24]. A rapid activation of arachidonic acid turnover in PI fraction was observed in feline adrenocortical cells in response to the cyclic AMP-mediated ACTH, and this effect seemed to be Ca^{2+}-dependent [25]. We are not aware of any literary data on the effect of hormonal stimulation on arachidonate metabolism in adrenal glomerulosa cells. Angiotensin II enhanced the incorporation of [^{3}H]arachidonate into PI. According to present views, arachidonate may be incorporated by acylating lyso-PI [26–28], or via CDP-DAG (Fig. 7) [29].

The conversion of PI into lyso-PI, was not increased by angiotensin, as examined by either incorporation or pulse-chase techniques. In addition, mepacrine failed to antagonize the stimulatory effect of angiotensin on [^{3}H]arachidonate incorporation into PI. These data show that the PI deacylation–

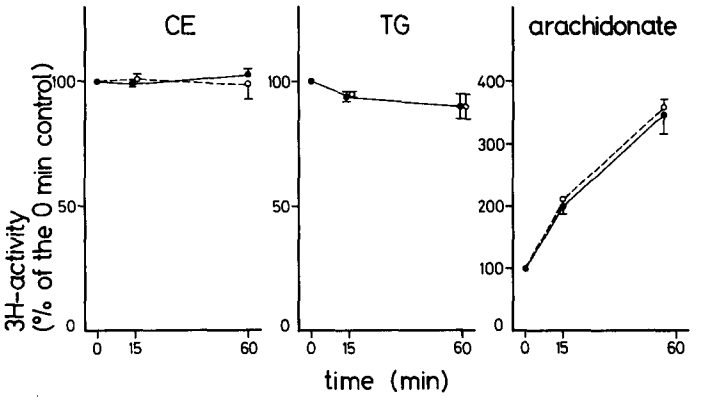

Fig. 6. Radioactivity of major lipid classes in glomerulosa cells prelabelled with [^{3}H]arachidonic acid. Cells were incubated in the presence of [^{3}H]arachidonate (120 MBq, 7 TBq/mmole) for 4 hr. After washing the cells twice, angiotensin II (25 nM) (--○--) or solvent (—●—) was added. This time is indicated as 0 min on the figure. Means ± S.E.M. of three separate experiments are shown, each carried out in duplicates. Radioactivity is expressed as per cent of the 0 min control. CE: cholesterol esters, TG: triacylglycerol.

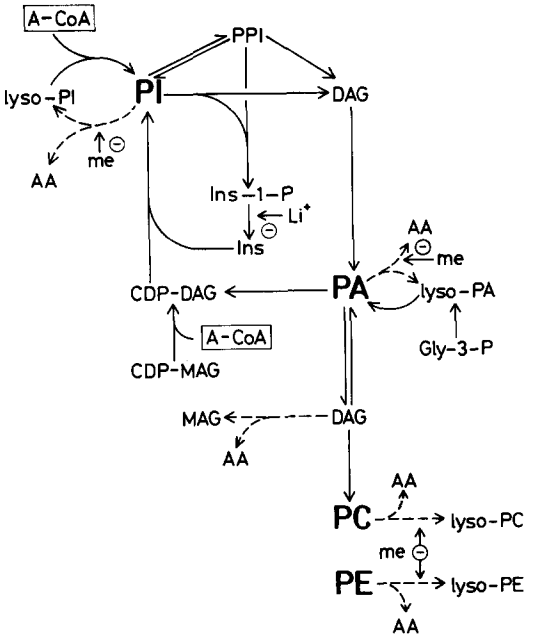

Fig. 7. Schematic representation of pathways involved in the metabolism of phospholipids. Metabolic routes resulting in arachidonate (AA) release are indicated with dotted arrows. Major phospholipid classes are emphasized by bold characters. Abbreviations not defined in the text: PPI, polyphosphoinositides; A-CoA, arachidonyl-CoA; Ins, inositol; Gly-3-P, glycerol-3-phosphate; me, mepacrine; MAG, monoacylglycerol.

reacylation cycle is not increased by this hormone in adrenal glomerulosa cells.

The observation that lithium abolished the effect of angiotensin on the incorporation of [^{3}H]arachidonate into PI suggests that hormone induced entry of labelled arachidonate takes place via CDP-DAG.

The only indication of arachidonate release in the present experiments might be the reduced incorporation of [^{3}H]arachidonate into PC and PE in response to angiotensin. Arachidonate may be released also from non-phospholipid sources. Our finding, however, that neither [^{3}H]arachidonate release nor the [^{3}H]labelling of cholesterol esters and triacylglycerols were significantly affected by angiotensin II failed to support this assumption. While all these latter data suggest the lack of any significant arachidonate release, it should be kept in mind that the size of the distinct lipid pools has been unknown. If the fractional breakdown of a large pool is relatively small, and is enhanced by angiotensin, the methods applied may fail to detect this effect, yet significant increase in free unlabelled arachidonate level may take place. Decrease in [^{3}H]labelling of PC in angiotensin-stimulated glomerulosa cells was accompanied by similar decrease in [^{32}P]incorporation. This decrease was reduced when angiotensin-induced increase in the synthesis of PI was prevented by lithium (Fig. 3). These data together raise the possibility that the decreased [^{3}H]labelling of PC and PE reflects a decrease in their synthesis due to substrate depletion (e.g. diacylglycerol). Such a substrate depletion may give support to the contention that in addition to stimulating the breakdown of PI (Fig. 5), angiotensin II also activates an enzyme participating in its synthesis, or inhibits phosphatidate phosphohydrolase.

The present results suggest that acute stimulation with angiotensin does not lead to the activation of phospholipase A_2. The only literary data favoring the role of phospholipase A_2 activation in the zona glomerulosa cells is that mepacrine abolishes the stimulatory effect of angiotensin on aldosterone production [30]. In this context, however, several nonspecific effects of phospholipase A_2 inhibitors [27, 31–33 etc.] may be recalled. Inhibition of calmodulin by mepacrine [31] may lead to the inhibition of angiotensin-stimulated aldosterone production [34]. Previous studies reported that non-steroidal inhibitors of prostaglandin synthesis do not impair the aldosterone secretory response of glomerulosa cells to physiological stimuli [6, 34]. On the other hand, angiotensin II failed to stimulate the formation of prostaglandins and prostacyclins in glomerulosa cells [35–37]. Our present results are compatible with these previous findings but also question the possible role of other metabolites of arachidonate (e.g. leukotrienes) in stimulus-secretion coupling of adrenal glomerulosa cells.

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