Kinetic aspects of cycloheximide-induced reversal of adrenocorticotropic effects on steroidogenesis and adrenal phospholipids in vivo

(hormone action/cyclic AMP/ phospholipid metabolism)

ROBERT V. FARESE, MOHAMMAD A. SABIR, AND RONALD E. LARSON

James A. Haley Veterans Hospital and Department of Internal Medicine, College of Medicine, University of South Florida, Tampa, Florida 33612

Communicated by George K. Davis, September 10, 1980

ABSTRACT Intraperitoneal injection of maximally effective doses of corticotropin (1-24) [ACTH(1-39)] provoked maximal increases in rat adrenal phospholipids as follows: phosphatidic acid within 1.5-2 min, phosphatidylinositol and phosphatidilylglycerol within 4-6 min, and polyphosphoinositides and corticosterone within 5-15 min. Continued maximal adrenal stimulation by ACTH (1-18) treatment caused sustained increases in adrenal phosphatidic acid, phosphatidylinositol, phosphatidilylglycerol, polyphosphoinositides, and corticosterone. Treatment with cycloheximide during this steady-state caused rapid decreases in all of these substances to basal levels. The observed half-lives of adrenal phosphatidic acid, phosphatidylinositol, polyphosphoinositides, phosphatidilylglycerol, and corticosterone during cycloheximide inhibition were 0.15, 1.0, 1.7, 3.3, and 3.5 min, respectively. Calculated production rates during maximal ACTH stimulation were 1000, 991, 90, 34, and 41 nmol/g of tissue per min, respectively. These findings suggest that (i) an initial effect of ACTH on de novo synthesis of phosphatidic acid can account for all subsequently observed increases in other phospholipid derivatives of CDP-diacylglycerol, (ii) a labile protein is required for the ACTH-induced increase in phosphatidic acid, (iii) the phosphatidate-polyphosphoinositol-polyglycerophospholipid pathway is rapidly and dramatically responsive to hormonal stimulation, (iv) changes in steroidogenesis correlate well with changes in this phospholipid pathway, and (v) stimulation of this pathway is rapidly reversible.

Corticotropin (ACTH) increases adrenal polyphosphoinositides (1), which, by virtue of their polyphosphorylated polar head groups, stimulate mitochondrial pregnenolone synthesis (2), the rate-determining step in steroidogenesis (3). We have shown (4) that (i) adrenal polyphosphoinositide concentrations correlate closely with steroidogenesis in various experimental conditions and (ii) cycloheximide, a protein-synthesis inhibitor that blocks ACTH effects on steroidogenesis (5), also inhibits new effects of ACTH on adrenal polyphosphoinositides and rapidly reverses such on-going effects. We also have observed in in vitro studies (6) that the ACTH-induced increase in polyphosphoinositides is preceded by increases in their precursors, phosphatidylinositol and phosphatic acid, and that cycloheximide also blocks these effects of ACTH. Our results suggested that ACTH rapidly increases adrenal phosphatidic acid by a cycloheximide-sensitive process and that this leads to rapid increases in mono- and polyphosphoinositides and other derivatives of CDP-diacylglycerol (e.g., phosphatidilylglycerol).

In this study we examined the rates of increase and decrease of adrenal phosphatidic acid, the inositides, phosphatidilylglycerol, and steroidogenesis during stimulation by ACTH and inhibition by cycloheximide in vivo. The latter treatment provided a unique opportunity to determine turnover and production rates of adrenal phospholipids in vivo, and it appears that phosphatidic acid turnover is extremely rapid during ACTH stimulation, whereas turnover rates of the inositides, phosphatidilylglycerol, and steroidogenesis are only slightly less rapid. Our findings suggest that (i) ACTH rapidly increases phosphatidic acid synthesis by a cycloheximide-sensitive process; (ii) the increase in phosphatidic acid underlies the subsequent rapid increase in all glycerolipids derived from CDP-diacylglycerol, including the polyglycerophospholipids and the polyphosphoinositides; and (iii) polyphosphoglycerolipids may be related to steroidogenesis.

MATERIALS AND METHODS

Male rats weighing approximately 200–250 g were obtained from Holtzman, Madison, WI, and housed in a light- and temperature-controlled environment for 1–2 wk before experimental use. Rats were injected with maximally effective doses of ACTH-(1-24) (two units) intraperitoneally or ACTH-(1-18) (10 units) intramuscularly to produce a rapid but transient [maximal for 20–30 min (4)] or long-lasting (maximal for 24–36 hr (7)] effect, respectively, on steroidogenesis. As suggested by Garren et al. (5), 10 mg of cycloheximide was injected intraperitoneally to produce almost immediate and total suppression of adrenal protein synthesis. In most experiments rats were killed by decapitation, and adrenals were removed (as rapidly as possible) 50–60 sec later and rapidly chilled by immersion in ice-cold physiological saline. In some experiments in which it was essential to remove adrenals even more quickly after cycloheximide injection, the rats were anesthetized for 10–15 min by intraperitoneal injection of 40–50 mg of pentobarbital per kg of body weight, and (without decapitation) both adrenals were simultaneously removed 15 sec after the abdominal incision. (The latter procedure did not influence the effectiveness of intraperitoneal cycloheximide.)

Adrenals were trimmed free of surrounding fat, weighed, and homogenized in 250 mM sucrose solution containing 50 mM Tris (pH 7.5). Tissues were kept at 0–4°C during all procedures. Small aliquots of the homogenate were taken for corticosterone determination [acid-fluorescence method (8)], and the phospholipids in the remainder were extracted as described (1). Phospholipids were purified by thin-layer chromatography with solvent system B as described (1) [successive unidimensional development with chloroform/methanol/4.3 M NH₂OH, 90:65:20 (vol/vol), followed by n-propanol/4.3 M NH₂OH, 65:35 (vol/vol), containing 10 mM cyclohexane-aminetraacetic acid] or with solvent system C [chloroform/pyridine/formic acid, 50:30:7 (vol/vol)]. In system B, the Rf values for phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine, phosphatidic acid, and phosphatidylserine, and triphospho-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: ACTH, corticotropin; t₁/₂, half-life.

7189
Acid respectivly. Chromatography, and phosphatidylcholine. Determined only aminated systems. Recoveries phosphoinositide it had been separated. Recoveries of all phospholipids were approximately 50% and were not influenced by treatments. Other methods for chromatography, lipid localization, and quantification of phosphorus by colorimetry have been described (1).

Both ACTH preparations were kindly provided by Organon. Cycloheximide and phospholipid standards were obtained from Sigma. Thin-layer chromatography plates (silica gel with 5% magnesium acetate) were obtained from Supelco, Bellefonte, PA.

RESULTS

Adrenal phosphatidic acid increased rapidly to maximal or near-maximal levels within 2 min of ACTH(1-24) injection (Fig. 1). In other experiments not shown, maximal levels were attained within 1.5 min (including 30 sec after injection and 60 sec between decapitation and adrenal removal). Phosphatidylglycerol and phosphatidylinositol increased in parallel slightly more slowly and reached maximal levels after 4-6 min.

![Fig. 1. Rapid effects of ACTH(1-24) on adrenal corticosterone](image)

FIG. 1. Rapid effects of ACTH(1-24) on adrenal corticosterone (○), diphosphoinositide plus triphosphoinositide (●), phosphatidylglycerol (▲), phosphatidylinositol (●), and phosphatidic acid (●). Mean results ± SEM of 3-6 determinations are shown (n = 3 for corticosterone and all inositides; n = 6 for phosphatidylglycerol and phosphatic acid). For each determination adrenals from 1-2 rats were used. In repeat experiments maximal values for polyphosphoinositides were observed between 5-15 min.

Polyphosphoinositides increased gradually over the course of this experiment, and there was remarkable parallelism between the production of these substances and adrenal corticosterone. The timing of the peak response of polyphosphoinositides varied somewhat from experiment to experiment, ranging from 5 to 15 min (4), but in all cases either preceded or coincided with the peak response of adrenal corticosterone.*

Long-acting ACTH(1-18) treatment evoked sustained maximal increases in steroidogenesis (adrenal corticosterone), polyphosphoinositides, phosphatidylglycerol, phosphatidylglycerol, and phosphatic acid, and nearly steady-state conditions prevailed between 60 and 180 min (Fig. 2).

When cycloheximide was injected into rats previously treated for 60 min with long-acting ACTH(1-18) (Fig. 3), adrenal corticosterone returned to the control range after 20 min, and inositides and phosphatic acid returned to control or lower levels by 6 and 2 min, respectively. On the other hand, phosphatidylserine, a derivative of phosphatidylethanolamine, was metabolically stable and did not decrease with cycloheximide treatment. In other experiments (not shown) phosphatidylglycerol decreased to basal over a 6-min period.

Fig. 4 shows the semilogarithmic plot of data from Fig. 3. The log of the concentration of the designated substances (expressed as the percentage of the ACTH-stimulated increase over the final basal level) decreased linearly with time. The half-lives

* The results of the experiment shown in Fig. 1 were selected for illustration because (i) polyphosphoinositides were simultaneously determined with other depicted phospholipids from the same adrenal tissues, (ii) early attainment of plateau levels of phosphatic acid and phosphatidylglycerol with continued increase in polyphosphoinositides suggests a precursor-product relationship, and (iii) the continued increase in steroidogenesis clearly coincides with changes in polyphosphoinositides rather than changes in other depicted phospholipids.
time of cycloheximide, was required to remove the adrenals. Mean ± SEM of six experiments are shown. Shaded area and open symbols indicate mean ± SEM of substances from control adrenals. See Figs. 1 and 2 for other details.

(t1/2) of serum corticosterone, adrenal corticosterone, and adrenal polyphosphoinositides were 10, 3.5, and 1.7 min, respectively. From these values (Table 1), the fractional turnover rate and total turnover rate at zero-time cycloheximide treatment could be calculated. The latter is equal to the production rate during maximal ACTH stimulation because steady-state conditions prevailed.

To obtain earlier time points for decay of phosphatidylserine and phosphatidic acid during cycloheximide blockade, experiments were conducted with more frequent sampling during the first 3.5 min. Phosphatidic acid, phosphatidylserine, and phosphatidyglycerol decreased curvilinearly during this time period. When plotted semilogarithmically, the decreases followed first-order kinetics, the t1/2 being approximately 0.15, 1, and 3.3 min, respectively (Fig. 5; see Table 1 for fractional turnover and production rates). Phosphatidylserine, on the other hand, did not change appreciably during this time period (this serves as an excellent internal control during sample workups). Adrenal corticosterone decreased only mildly in these experiments (results not shown). Results of phosphatidic acid determinations in Fig. 5 were derived from two experimental protocols, one with pentobarbital anesthesia and laparotomy (results at 30–90 sec) and one without anesthesia (results at 90–210 sec). In both, basal levels of phosphatidic acid were achieved by 90 sec of cycloheximide treatment, and apparently the anesthesia and laparotomy did not influence the decay process appreciably. Also, upon extrapolation back to 100% ACTH stimulation, the onset of the decrease of phosphatidic acid was approximately 30 sec after intraperitoneal injection of cycloheximide, and this preceded the apparent onset of decrease for phosphatidylserine and phosphatidyglycerol.

The calculated production rate (Table 1) for phosphatidic acid (1060 nmol/g of tissue per min) is virtually the same as the combined production rates of phosphatidylserine and phosphatidyglycerol, two major derivatives of CDP-diacylglycerol. This agreement suggests that the observed t1/2 for phosphatidic acid during cycloheximide blockade is not underestimated.

**DISCUSSION**

The results confirm our earlier findings in *in vitro* studies (6) that ACTH-induced increases in concentrations of adrenal polyphosphoinositides are preceded or accompanied by net increases in phosphatidylserine, phosphatidyglycerol, and phosphatidic acid. Laychock *et al.* (10) also observed a net increase in phosphatidylserine after 90 min of ACTH treatment of cat adrenal cells in *vitro*, but levels of phosphatidic acid were unchanged. From our results, it appears that ACTH stimulates the entire phosphatidate—polyphosphoinositide—polyglycerophospholipid pathway, and the present *in vivo* kinetic data coupled with those obtained in *vitro* (4, 6) strongly suggest that

---

1 It should be recognized that (i) corticosterone is the end-product of a steroidogenic pathway in which the first step (namely, cholesterol side-chain cleavage) is rate-limiting and stimulated by a "steroidogenic factor" that enhances substrate binding to cytochrome P-450 (9), and (ii) the t1/2 of adrenal corticosterone is dependent on adrenal secretion (or washout) and decreased synthesis. Thus, the slight difference between t1/2 for adrenal corticosterone and that for polyphosphoinositides cannot be taken as evidence against the hypothesis that the polyphosphoinositides are steroidogenic factors in ACTH action. On the contrary, such a slight difference in the direction observed is not unexpected in the context of this hypothesis and the above considerations.
ACTH, through cyclic AMP, increases de novo synthesis of phosphatidic acid, either from fatty acyl-CoA and sn-glycerol-3'-PO₄ or from a nonphospholipid source of 1,2-diacylglycerol and ATP. This suggestion (regarding a de novo increase) seems likely because ACTH also increases phosphatidylcholine and all or most phospholipids by a cycloheximide-sensitive process (11); therefore, it is improbable that ACTH increases phosphatidate at the expense of a decrease in other phospholipids or 1,2-diacylglycerol.²

The rapidity of the increase in phosphatidic acid and derivatives of CDP-diacylglycerol after ACTH treatment in vivo is remarkable. Phosphatidic acid is maximal within 1.5–2 min, phosphatidylglycerol and phosphatidylinositol are maximal at 4–6 min, and polyphosphoinositides are maximal at 5–15 min. Steroidogenesis appears to parallel or to follow the increase in polyphosphoinositides. The same sequence is observed in incubations of adrenal quarters (6), but more time (2- to 3-fold) is required to achieve maximal levels of phospholipids and steroidogenesis in vitro.

The ACTH-induced, steady-state increases in adrenal phospholipids and corticosterone and their first-order decay rates during the cycloheximide blockade provided a unique opportunity to estimate their fractional turnover and production

---

### Table 1. Turnover and production rates of corticosterone and adrenal phospholipids during ACTH stimulation and cycloheximide blockade in vivo

<table>
<thead>
<tr>
<th>Substance</th>
<th>Labile adrenal concentration, nmol/g of tissue</th>
<th>Half-life, min</th>
<th>Fractional turnover, nmol/g of tissue per min</th>
<th>Maximal production rate, nmol/g of tissue per min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum corticosterone</td>
<td>—</td>
<td>10.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Adrenal corticosterone</td>
<td>206</td>
<td>3.5</td>
<td>0.20</td>
<td>41</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>230</td>
<td>0.15</td>
<td>4.62</td>
<td>1060</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>1436</td>
<td>1.0</td>
<td>0.69</td>
<td>991</td>
</tr>
<tr>
<td>Polyphosphoinositides</td>
<td>83</td>
<td>1.7</td>
<td>0.41</td>
<td>34</td>
</tr>
<tr>
<td>Phosphatidylglycerol</td>
<td>428</td>
<td>3.3</td>
<td>0.21</td>
<td>90</td>
</tr>
</tbody>
</table>

Half-lives ($t_{1/2}$) were derived from Figs. 3–5. Fractional turnover ($K$) = $0.693/t_{1/2}$. Maximal ACTH-stimulated production rate = $K \times$ (concentration at 60-min-ACTH and 0-min-cycloheximide treatments).

* That concentration decreasing with cycloheximide treatment in ACTH-stimulated adrenals (data from Figs. 3–5)

---

¹ Alternatively, it is possible that ACTH could increase (and cycloheximide decrease) the conversion of "metabolically inactive" 1,2-diacylglycerol to phosphatidic acid and use of the phosphatidate → polyphosphoinositol-polyglycerophospholipid pathway to generate "metabolically active" 1,2-diacylglycerol and ultimately phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine.

---

**Fig. 5.** Effects of cycloheximide on adrenal phosphatidylserine (X), phosphatidylglycerol (○), phosphatidylinositol (□), and phosphatidic acid (◆) during ongoing (as in Fig. 3) ACTH(1–18) (×60 min) stimulation. Two separate experiments were conducted: one to obtain values for each of the four phospholipids at times between 90 and 210 sec of cycloheximide treatment; the other for values of phosphatidic acid between 30 and 90 sec. In the former, the rats were decapitated 60 sec before the time of actual adrenal removal; in the latter, the rats were anesthetized 10–15 min before cycloheximide injection, and the abdomen was opened 15 sec before the time of adrenal removal. Shown on the abscissa is the total elapsed time between intraperitoneal cycloheximide injection and adrenal removal. Each experimental value is the mean of two or three determinations. *(Left)* Percentage of the ACTH-stimulated increases in designated substances over the final or basal concentrations after cycloheximide-induced decay. *(Right)* Semilogarithmic plot of data. Broken lines indicate extrapolations. ($C - C_0$)/(C₀ - Cₙ), Concentration at designated time = final concentration/initial concentration = final concentration. $t_{1/2}$: phosphatidylglycerol, 3.3 min; phosphatidylinositol, 1 min; phosphatidic acid, 0.15 min.
rates. From these data (Table 1), many points seem clear. First, of the substances studied, phosphatidate has the most rapid turnover and is rapidly converted to CDP-diacylglycerol (which does not accumulate) and its derivatives. Second, single effects of ACTH and cycloheximide on phosphatidic acid synthesis could account for all observed effects on other phospholipids. Third, most phospholipids in the phosphatidate→polyphosphoinositide-polyglycerophospholipid pathway are labile, and turnover is sufficiently rapid to be related to steroidogenesis. Fourth, there appears to be at least two pools for most phospholipids in this pathway: one that rapidly increases and decreases during the treatment with ACTH and cycloheximide, respectively, and another that is relatively stable [note the asymptotes during continued cycloheximide blockade; also note that cycloheximide does not decrease phospholipid levels in control tissues (4, 6)]. Fifth, most CDP-diacylglycerol is converted to inositol lipids rather than to polyglycerophospholipids. Sixth, most phosphatidylglycerol is converted to substances other than polyphosphoinositides (perhaps 1,2-diacylglycerol).

Seventh, phosphatidylglycerol metabolism to 1,2-diacylglycerol does not lead to substantial resynthesis of phosphatidic acid during cycloheximide blockade, and this suggests involvement of labile protein in this resynthesis. Eighth, phosphatidylglycerol phosphate and cardiolipin, both potentially steroidogenic (2), are probably increased during ACTH treatment. Inhibitory effects of cycloheximide on the phosphatidate→polyphosphoinositide-polyglycerophospholipid pathway may be explained variously. First, cycloheximide may cause nonspecific effects on phosphatidate synthesis, degradation, or measurement. However, this seems unlikely because puromycin, a distal protein synthesis inhibitor, also inhibits ACTH effects on this phospholipid pathway, and cycloheximide does not decrease basal levels (or measured turnover through analyses) of phospholipids in the pathway (4, 6). Second, phosphatidate and protein synthesis may be tightly coupled [e.g., in bacterial systems, inhibition of protein synthesis causes accumulation of ppGpp, which inhibits phosphatidate synthesis (11, 13)]. Third, a labile protein may be required in the stimulation of phosphatidate synthesis by ACTH and cyclic AMP. Although the last possibility seems most likely, the extreme rapidity of decrease in phosphatidate during cycloheximide blockade would require postulation that this protein functions and is inactivated almost immediately after its synthesis. In view of the rapidity of increase in phosphatidate after ACTH treatment, it seems unlikely that ACTH would increase the synthesis of this putative protein.

The attainment of steady-state increases in the concentrations of phospholipids in the phosphatidate→polyphosphoinositides→polyglycerophospholipid pathway during continued ACTH stimulation indicates that the production rates for these phospholipids are balanced by their degradation rates. Accordingly, a considerable fraction of phosphatidylglycerol (see Table 1) is probably converted to 1,2-diacylglycerol, which may be utilized for phosphatidate resynthesis (14, 15) or synthesis of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine. Thus, a generalized increase in phospholipids may follow, and this could trigger subsequent adrenal hypertrophy. In support of this, phospholipid, protein, and RNA synthesis are tightly coupled in bacteria (11, 13), and cycloheximide (possibly through effects on phospholipid or protein synthesis) inhibits the ACTH-induced increase in adrenal RNA polymerase (16).

The observed ACTH-induced increases in the concentrations of phospholipids in the phosphatidate→polyphosphoinositide-polyglycerophospholipid pathway are considerably different from those that accompany increases in phosphatidylinositol breakdown. The latter "phospholipid effect", long recognized (14, 15, 17), increases phosphatidic acid and polyglycerophospholipids but decreases phosphatidylinositol and the polyphosphoinositides and does not cause net increases in total phospholipids. These differences may be important because inositides, particularly the polyphosphoinositides, bind Ca2+ (18, 19) and decrease membrane fluidity (20). Consequently, a number of membrane properties may be altered, and the potential for metabolic regulation seems clear.

Parathyroid hormone induces changes in kidney cortex phospholipids (21) that are virtually identical to those induced by ACTH in the adrenal cortex [i.e., stimulation through cyclic AMP of the phosphatidate→polyphosphoinositide-polyglycerophospholipid pathway by a cycloheximide-sensitive mechanism (ref. 21 and unpublished observations)]. Since ACTH and parathyroid both utilize cyclic AMP as a "second messenger" (22–24), stimulation of the phosphatidate→polyphosphoinositide-polyglycerophospholipid cycle may be a frequent concomitant in cyclic AMP action. In addition, an extremely labile protein may be present in many tissues and may play a pivotal role in phosphatidic acid synthesis and subsequent metabolic regulation during the action of hormones, neurotransmitters, and other agents.

In support of this contention, we also have observed (unpublished observations) that cycloheximide blocks the net increase in phosphatidic acid that occurs subsequent to phosphatidylinositol breakdown (cycloheximide does not diminish the latter) during stimulation of rat pancreas with carbamoylcholine and other secretagogues.