Comparison of Changes in Inositide and Noninositide Phospholipids during Acute and Prolonged Adrenocorticotropic Hormone Treatment in Vivo†

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ABSTRACT: To further evaluate the potential role of phospholipids in the steroidogenic action of adrenocorticotropic hormone (ACTH), we compared ACTH-induced increases in rat adrenal inositide and noninositide phospholipids during acute (1–4 h) and more prolonged (48 h) ACTH treatment in vivo. With acute ACTH treatment, peak levels of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were achieved within 15 min, and this correlated reasonably well temporally with increases in phosphatidylinositol (PI) and polyphosphoinositides (PPI) observed presently and previously. After 48 h of ACTH treatment, the increases in adrenal concentrations of PE, PC, PI, PPI, and phosphatidic acid (PA) were virtually the same as those observed with acute ACTH treatment. With both acute and more prolonged ACTH treatments, PA, PI, and PPI increased nearly 2-fold, whereas PC and PE increased only 25–45%. Cycloheximide administration caused ACTH-induced increases in PC and PE to rapidly return to (or toward) control levels, and the rates of decrease in these phospholipids were only slightly slower than those observed for PI, PPI, PA, and adrenal corticosterone. The rapidity of cycloheximide-induced decreases in steroidogenesis and all adrenal phospholipids was similar in acute and prolonged ACTH treatment. From these findings, we conclude the following: (a) major adrenal phospholipid changes are similar in acute and prolonged ACTH treatment; (b) percent increases in inositides are greater than those of noninositide phospholipids during acute and prolonged ACTH treatment; (c) ACTH-induced increases in adrenal noninositide phospholipids are as dependent upon protein synthesis as are increases in the inositides; (d) protein synthesis dependence of induced increases in adrenal steroidogenesis and phospholipids is equally apparent in acute and prolonged ACTH treatment; and (e) although the present findings continue to support the possibility that inositides (?PPI) may play an important role in the steroidogenic action of ACTH, it now seems clear that noninositide phospholipids may also participate in this action.

We have previously reported that adrenocorticotropic hormone (ACTH) rapidly increases the concentrations of adrenal phospholipids in the phosphatidate–inositide pathway (Farese et al., 1979, 1980a–c). This stimulatory effect of ACTH is apparently mediated by adenosine cyclic 3′,5′-phosphate (cAMP), which increases de novo phosphatidate synthesis by a Ca^{2+}-dependent, cycloheximide-inhibitable process (Farese et al., 1979, 1980a–c, 1981). Presumably, these or related changes in phospholipids enhance steroidogenesis (Farese et al., 1979, 1980a–c, 1981; Farese & Sabir, 1979, 1980) by virtue of their ability (Farese & Sabir, 1979, 1980; Kido et al., 1979; Hanukoglu et al., 1981) to influence cytochrome $P\text{-}450_{sso}$ or the interaction of cholesterol with the latter enzyme and/or subsequent cholesterol side-chain cleavage (Jefcoate et al., 1970; Simpson et al., 1972; Brownie et al., 1973), which is rate limiting for steroidogenesis.

In stimulating de novo phosphatidate synthesis, ACTH would also be expected to increase the levels of phospholipids that are extrinsic to the phosphatidate–inositide pathway. This was in fact shown in in vitro studies (Farese et al., 1981) where ACTH and cAMP increased the levels of, and [^{3}H]glycerol incorporation into, phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Since these phospholipids are the most plentiful of adrenal phospholipids, it seemed important to more fully characterize their increases during both acute and more prolonged ACTH treatments in vivo and to compare these to changes in inositides and steroidogenesis. In addition, it seemed important to compare the inhibitory effects of cycloheximide on inositide and noninositide phospholipids during acute and prolonged ACTH stimulation in vivo. Such studies not only would provide a more complete assessment of ACTH-induced phospholipid changes in the intact animal but also would yield information that may be important in relating changes in phospholipids to other actions of ACTH, most notably, steroidogenesis, during acute and more prolonged stimulation.

Experimental Procedures

Male rats (250 g) were obtained from the Holtzmann Co. and housed in environmentally controlled quarters for 1–2 weeks prior to experimental use. $ACTH^{1-24}$ (2 units) and $ACTH^{1-18}$ (10 units) (both ACTH preparations were kindly provided by Organon) were injected intraperitoneally and intramuscularly to provoke immediate and long-acting (up to 24–48 h) effects, respectively, on adrenal metabolism [see Farese et al. (1980a–c)]. In some experiments, 10 mg of cycloheximide was injected intraperitoneally to rapidly reverse ACTH-induced increases in corticosterone and phospholipids [see Garren et al. (1965) and Farese et al. (1980c)]. Control rats were not injected since this might provoke endogenous ACTH release. Adrenals were rapidly removed from carcasses and analyzed for corticosterone, protein, and phospholipids by techniques described previously (Farese et al., 1979, 1980a–c, 1981). Protein concentrations (i.e., milligrams of protein per wet weight of tissue) were not altered by acute or chronic ACTH treatment.

In some experiments, adrenal zona fasciculata-reticularis cells were dispersed by treatment of decapsulated adrenals with collagenase and DNase, as described previously (Farese et al., 1981). Aliquots of cell suspensions were incubated at 37 °C under 95% O_2 plus 5% CO_2 in 1 mL of Kreb's Ringer bicarbonate buffer containing 10 mM glucose and 4% bovine serum albumin (Sigma). Other details of the incubation are described in Figure 4. After incubation cells and media were separated by centrifugation and analyzed for phospholipids and corticosterone, respectively.

After extraction, phospholipids were purified by thin-layer chromatography, with solvent system B for phosphatidylinositol

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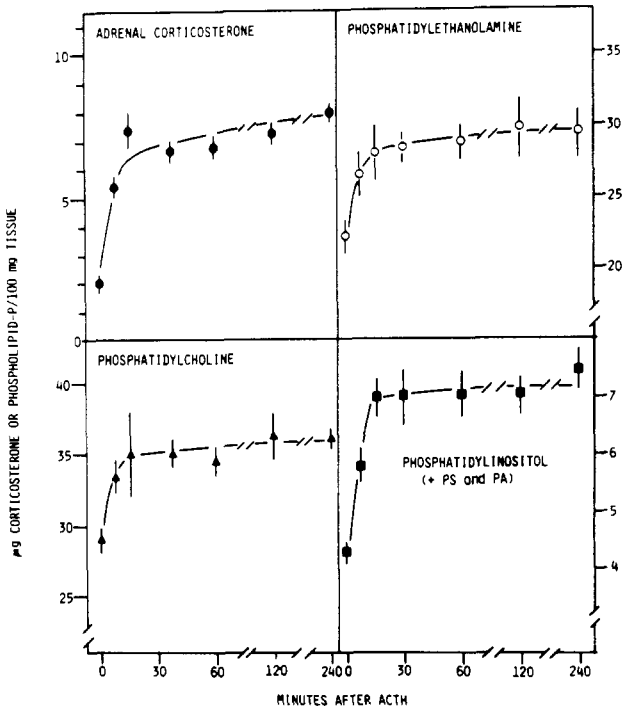

FIGURE 1: Time course of acute ACTH-induced increases in adrenal concentrations of corticosterone (●), phosphatidylethanolamine (○), phosphatidylcholine (▲), and phosphatidylinositol [plus phosphatidylserine (PS) and phosphatidic acid (PA)] (■). Rats were injected with both $ACTH^{1-24}$ and $ACTH^{1-18}$ to ensure rapid and sustained maximal adrenal stimulation (see Experimental Procedures). Phospholipids were purified by thin-layer chromatography with solvent system D. Shown here are mean values ± SEM of six determinations.

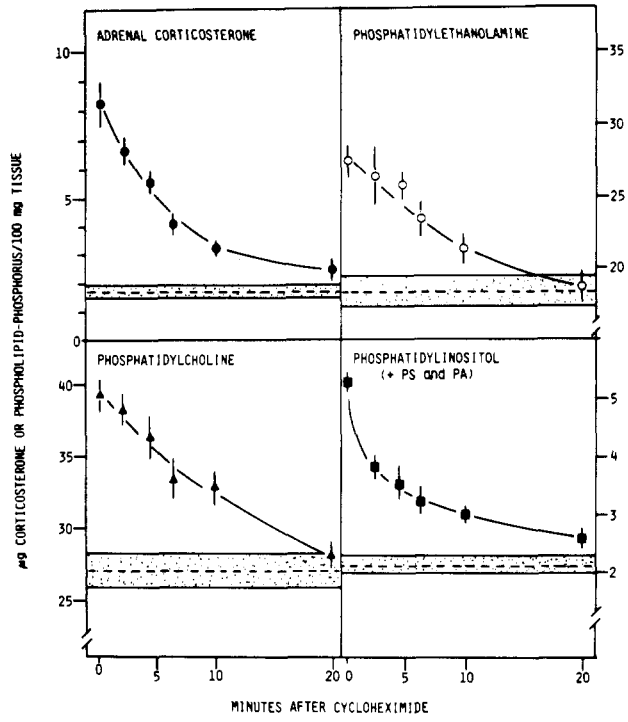

FIGURE 2: Cycloheximide-induced reversal of acute ACTH effects on adrenal concentrations of corticosterone (●), phosphatidylethanolamine (○), phosphatidylcholine (▲), and phosphatidylinositol [plus phosphatidylserine (PS) and phosphatidic acid (PA)] (■). Rats were treated with $ACTH^{1-24}$ and $ACTH^{1-18}$ as described in Figure 1 to provoke rapid and sustained maximal adrenal stimulation. After 1 h of such ACTH treatment, 10 mg of cycloheximide was injected intraperitoneally to rapidly reverse ACTH effects on steroidogenesis and phospholipid metabolism [see references cited in Farese et al. (1980a–c) and Garren et al. (1965)]. Phospholipids were purified by thin-layer chromatography with solvent system D. Shown here are the mean values ± SEM of six determinations. The shaded areas depict the mean values ± SE for control (uninjected) rats.

(PI), phosphatidic acid (PA), and polyphosphoinositides [see Farese et al. (1980a–c)] and with solvent system D (Farese et al., 1981) for PC and PE. In the latter system, for comparative purposes, an area of the chromatogram containing PI, phosphatidylserine (PS), and PA was also analyzed; changes in this area primarily reflected changes in PI, since the ratio of these three phospholipids in ACTH-stimulated tissues was approximately 1/0.16/0.1, and PS was only minimally affected by ACTH and cycloheximide treatment (Farese et al., 1980a–c).

Results

Shown in Figure 1 are the acute ACTH-induced changes in adrenal concentrations of corticosterone, PE, PC, and PI (plus PS and PA). Maximal or near-maximal effects of ACTH on all substances were observed within 15 min of treatment, although less-than-maximal effects were significantly different from control at the earliest time of sampling, viz., 7.5 min. Maximal effects of ACTH on all substances persisted throughout the 4-h observation period. (Note: there were no changes in adrenal weight or protein content or concentration in this time period.) It may be noted that PI increased approximately 75–100% [also see Figure 2 and Farese et al. (1980a–c)], while PC and PE increased only 25–45%; further, the absolute increments in each of the latter two phospholipids were greater than that of PI [6–12 vs. 3–4 μg of phospholipid phosphorus/100 mg of tissue (wet weight)]. Although not shown in Figure 1, serum corticosterone increased from 5 to 40 μg/dL over the first 30 min and remained constant thereafter.

Administration of cycloheximide to rats previously treated for 1 h with ACTH led to rapid reversal of ACTH-induced increases in steroidogenesis (adrenal corticosterone), PI (plus PS and PA),[1] PC, and PE (Figure 2). As is apparent, PC and PE decreased at rates that were only slightly less than that of corticosterone and PI (plus PS and PA).

Although acute ACTH-induced increases (less than 2–4 h) in adrenal phospholipids in vivo have been well described previously [see Farese et al. (1979, 1980a–c) and above], there is virtually no information on more prolonged effects of ACTH on adrenal phospholipid metabolism. We therefore treated rats with long-acting $ACTH^{1-18}$ for 48 h and examined adrenal phospholipid levels. In these experiments, adrenal wet weight increased approximately 40–50% [from 43 ± 2 to 61 ± 4 (mean ± standard error; $n = 4$) mg/adrenal pair] after ACTH treatment, protein content increased proportionally so that protein concentration was unchanged, and serum corticosterone levels were markedly elevated (40–70 vs. 3–10 for controls and 20–40 μg/dL for rats treated for 1 h with ACTH). In addition, as shown in Table I, after 48 h of ACTH treatment, adrenal concentrations of polyphosphoinositides, PA, and PI increased approximately 2-fold, and PC and PE concentrations increased approximately 40%: of note, these changes in phospholipid concentrations were virtually the same as those observed with more acute ACTH treatment [see above and Farese et al. (1979, 1980a–c)].

Although cycloheximide is known to rapidly reverse increases in steroidogenesis and phospholipids during acute ACTH treatment (less than 2 h) [see above, Farese et al.

[1] More detailed analysis of cycloheximide-induced changes in more highly purified preparations of phosphatidylinositol, phosphatidylserine, phosphatidic acid, and polyphosphoinositides has been reported previously (Farese et al., 1980c).

Table I: Effects of 48-h ACTH Treatment on Adrenal Phospholipid Concentrations[a]

phospholipid	control (ng of phospholipid P/100 mg of tissue)	ACTH treated (ng of phospholipid P/100 mg of tissue)	P (ACTH vs. control)
polyphosphoinositides	301 ± 27	766 ± 34	<0.001
phosphatidic acid	309 ± 21	533 ± 26	<0.001
phosphatidylinositol	4 221 ± 157	7 438 ± 486	<0.001
phosphatidylcholine	23 326 ± 2519	34 504 ± 561	<0.005
phosphatidylethanolamine	25 258 ± 2584	34 494 ± 514	<0.01

[a] Rats were injected intramuscularly with 10 units of long-acting $ACTH^{1-18}$ peptide in saline 48, 24, and 2 h prior to sacrifice. For each determination, adrenal glands from three rats were pooled and extracted. Two-thirds of the lipid extract was concentrated and chromatographed in solvent system B, providing results for polyphosphoinositides, phosphatidic acid, and phosphatidylinositol; the remaining one-third was concentrated and chromatographed in solvent system D, providing results for phosphatidylcholine and phosphatidylethanolamine. Mean values ± standard error of four determinations are shown. P was determined by standard t testing.

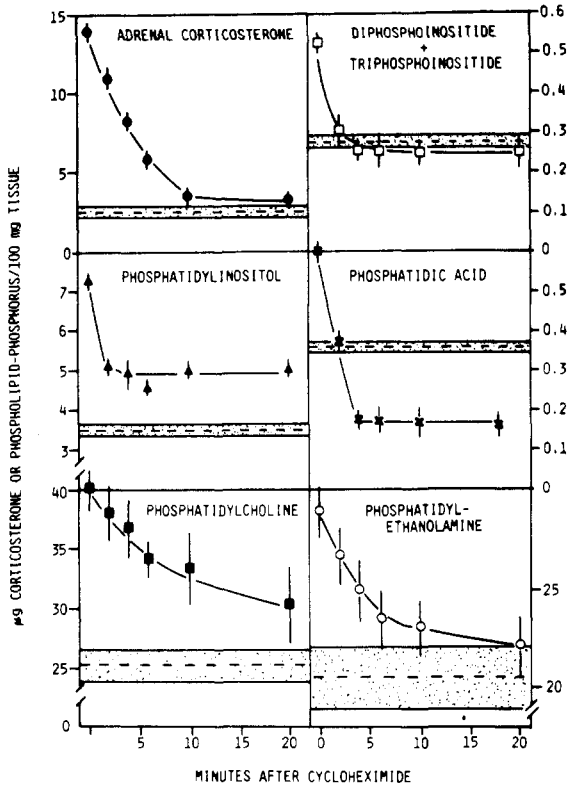

FIGURE 3: Cycloheximide-induced reversal of prolonged ACTH effects on adrenal concentrations of corticosterone (●), diphosphoinositide plus triphosphoinositide (□), phosphatidylinositol (▲), phosphatidic acid (×), phosphatidylcholine (■), and phosphatidylethanolamine (○). Rats were treated for 48 h with $ACTH^{1-18}$ as in Table I. Cycloheximide (10 mg) was given intraperitoneally as in Figure 2. Diphosphoinositide, triphosphoinositide, phosphatidylinositol, and phosphatidic acid were purified by thin-layer chromatography with solvent system B; for phosphatidylcholine and phosphatidylethanolamine, solvent system D was employed. Shown here are mean values ± SE of six determinations. The shaded areas depict the mean values ± SE for control (uninjected) rats.

(1980a–c), and Garren et al. (1965)], effects of cycloheximide on more prolonged ACTH effects have not been examined. We therefore administered cycloheximide to rats treated for 48 h with ACTH. As shown in Figure 3, cycloheximide rapidly reversed ACTH-induced increases in steroidogenesis and adrenal concentrations of polyphosphoinositides, PA, PI,

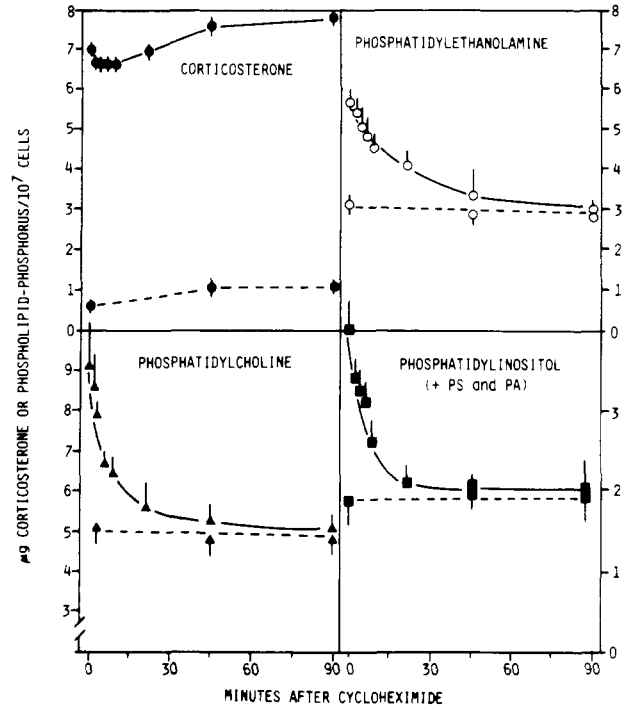

FIGURE 4: Effects of ACTH and cycloheximide on corticosterone production (●) and adrenal concentrations of phosphatidylcholine (▲) and phosphatidylethanolamine (○) in vitro. Adrenal cells were incubated without (---) or with (—) 10^{-6} M ACTH for 30 min. Cycloheximide (1 mM) was then added to ACTH-treated cells (in other experiments, cycloheximide did not affect control phospholipid concentrations) and incubation was continued for the indicated times. Each value is the mean ± SE of four determinations. [Note: in ACTH-stimulated cells that are not treated with cycloheximide, medium corticosterone continues to increase (accumulate) linearly for over 2 h, and increases in phospholipids are sustained; thus, the failure of corticosterone levels to increase further in ACTH plus cycloheximide-treated reaction mixtures indicates an immediate and sustained interruption of ACTH effects by cycloheximide.]

PC, and PE. These results were in fact quite similar to those observed with more acute ACTH treatment [see above and Farese et al. (1980c)]. Again, the rates of decrease of PC and PE were only slightly less than those of adrenal corticosterone, polyphosphoinositides, PI, and PA. It may also be noted that PI and PC did not return fully to control levels during cycloheximide treatment, whereas PA fell below the control level [also noted previously (Farese et al., 1980a–c)]. The former may be due to heterogeneity of phospholipid pools, and the latter may reflect a greater dependence of PA on its continued synthesis.

To be certain that effects of ACTH and cycloheximide treatments in the above in vivo experiments were directly due to these agents, and not to nonspecific or secondary effects, we conducted in vitro experiments. As shown in Figure 4, addition of ACTH to incubations of adrenal cells for 30 min provoked increases in PE and PC that were even greater than those observed in vivo (the reason for this is unknown). Addition of cycloheximide immediately blocked further increases in corticosterone production, and PC and PE returned gradually to basal levels.

Discussion

The present results from in vivo experiments complement those from in vitro experiments (Farese et al., 1981) and clearly indicate that ACTH increases the two major adrenal phospholipids, PC and PE, as well as the phospholipids in the phosphatidate–inositide cycle. The failure to observe increases in PC and PE in earlier experiments (Farese et al., 1979,

1980a–c) probably reflects the fact that our attention was initially focused on the more dramatic increases in inositides, and we did not routinely measure PC and PE; in addition, we did not cleanly separate the latter phospholipids by chromatography in solvent systems A–C, and the large amounts of phospholipids in the combined PC–PE area (solvent system B) may have caused less-than-optimal conditions for lipid-phosphorus quantitation. In any event, with employment of the present methods, most notably, solvent system D, the ACTH-induced increases in the PC and PE were clear.

Although PC and PE did not increase percentagewise as much as inositides after ACTH treatment, the absolute increases in concentrations of PC and PE were greater than those of the inositides. The inositides, however, have severalfold greater fractional turnover rates (Farese et al., 1980c) than PC and PE, and this offsets the greater ACTH-induced increases in PC and PE when production rates are calculated. Thus, from the data in Figures 2 and 3 and those obtained previously (Farese et al., 1980c), if the half-lives for *ACTH-induced increases* in PI, PC, and PE are accepted as 1.7, 6, and 7 min, respectively, and if ACTH-induced increments are 3, 12, and 9 μg of phospholipid phosphorus/100 mg of tissue, the rates of turnover [and synthesis, since ACTH-induced increases had attained a steady state (see Figure 1)] can be calculated (Farese et al., 1980c) to equal 1.2, 1.3, and 0.9 μg of phospholipid phosphorus (100 mg of tissue)$^{-1}$ min^{-1}, respectively, for the above phospholipids. It would thus appear that approximately one-third of ACTH-induced PA traverses the inositide pathway and two-thirds is metabolized to PC and PE.

It is of interest that the ACTH-induced increases in the concentrations of adrenal phospholipids are relatively constant during acute and more prolonged ACTH treatment. If these changes in phospholipids are important in steroidogenesis, further increases in adrenal steroidogenic activity during prolonged ACTH treatment (Holmes et al., 1980) would have to be explained by other factors, e.g., increases in adrenal weight, steroidogenic enzymes, or free cholesterol availability. Of further interest is the fact that prolonged ACTH-induced increases in steroidogenesis and adrenal phospholipids are as sensitive to cycloheximide as the more acute effects of ACTH thereon. Thus, regardless of the duration of the ACTH effect on steroidogenesis, the latter appears to require increases in adrenal phospholipids and continued operation of a labile protein.

It is somewhat surprising that ACTH-induced increases in PC and PE are nearly as labile as the increases in the inositides and phosphatidylglycerol (Farese et al., 1980c). Since cycloheximide decreases primarily the ACTH-induced increases in most phospholipids (and not the control levels), it seems likely that there is (are) phospholipid pool(s) that is (are) unique to the stimulated state, and this (these) pool(s) involve(s) both inositides and noninositide phospholipids. A remarkable characteristic of this (these) ACTH-induced phospholipid pool(s) is its rapid turnover and return to the basal state during cycloheximide-induced inhibition of de novo phospholipid synthesis.

Although PC and PE are increased by ACTH treatment, the greater percentage increase in inositides indicates that chemical properties of the latter will not be negated by increases in the former two phospholipids. Thus, relative enrichment of membranes with inositides would be expected to influence Ca^{2+} binding (Michell, 1975; Hawthorne & White, 1975; Hendrickson & Reinertsen, 1971; Buckley & Hawthorne, 1972), steroidogenesis (see above), and presumably other cellular functions. In addition, greater concentrations of PC may increase cholesterol esterase activity (Nishikawa et al., 1981), and greater concentrations and "flow" of all phospholipids may promote the transfer of lipid-soluble substances, e.g., cholesterol, between and across membranes and thus enhance the interaction of cholesterol with cytochrome P-450$_{scc}$. Thus, although previous studies (Farese et al., 1979, 1980a–c; Farese & Sabir, 1979, 1980) had implicated inositides in the steroidogenic effect of ACTH, the present documentation of rapid, parallel changes in noninositide phospholipids during ACTH and cycloheximide treatment raises the possibility that these phospholipids may also participate in the steroidogenic response.

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