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ACTH INCREASES *DE NOVO* SYNTHESIS OF DIACYLGLYCEROL AND TRANSLOCATES PROTEIN KINASE C IN PRIMARY CULTURES OF CALF ADRENAL GLOMERULOSA CELLS

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Summary—Effects of ACTH on the production of diacylglycerol (DAG) and translocation of protein kinase C were studied in primary cultures of calf adrenal glomerulosa cells. To study DAG production two different labeling protocols were used: (a) cells were prelabeled for 3 days with [2-^{3}H]glycerol before ACTH addition; (b) ACTH and [2-^{3}H]glycerol were added simultaneously to cells. In both cases, ACTH provoked rapid increases in the labeling of DAG which were maximal in 2 min, dose-dependent, and paralleled by increases in DAG mass. ACTH also increased the labeling of total glycerolipids including phosphatidic acid (PA), phosphatidylinositol, phosphatidylethanolamine, phosphatidylcholine and triacylglycerol. In both labeling protocols, the rates of increase in the labeling of DAG and PA were greater than those of other glycerolipids. Our results indicate that ACTH rapidly increases DAG, at least partly by stimulating the *de novo* synthesis of PA. In addition, we found that ACTH, like phorbol esters, stimulated the apparent translocation of immunoreactive protein kinase C from the cytosol to the membrane fraction.

INTRODUCTION

ACTH is known to provoke rapid increases in the levels of phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylinositol-4′-phosphate (PIP) phosphatidylinositol-4′,5′-biphosphate (PIP_2), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and diacylglycerol (DAG) in the adrenal cortex [1–6], without substantially altering the hydrolysis of PIP_2, PIP or PI by phospholipase C [7–9]. The mechanism whereby ACTH increases DAG is, however, poorly understood, and this may be important [10], since ACTH has been reported to activate protein kinase C [6, 11–13]. To gain further insight into the mechanisms whereby ACTH increases DAG the effects of ACTH on [^{3}H]glycerol incorporation into PA, DAG and other lipids were examined, both in acute labeling and chronic prelabeling experiments in primary cultures of calf adrenal glomerulosa cells. ACTH provoked rapid and sizeable increases in [^{3}H]glycerol labeling of PA, DAG and total glycerolipids using both labeling protocols, as well as DAG content. Our results indicate that ACTH increases DAG, at least partially, through enhanced synthesis of PA *de novo*. In addition, ACTH, like 12-*O*-tetradecanoyl phorbol-13-acetate (PMA) [14], induced the translocation of immunoreactive protein kinase C from the cytosol to the membrane fraction.

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Abbreviations: ACTH, adrenocorticotropin; PA, phosphatidic acid; PI, phosphatidylinositol; PIP, phosphatidylinositol-4′-phosphate; PIP2, phosphatidylinositol-4′,5′-bis-phosphate; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PL, phospholipids; MAG, monoacylglycerol; DAG, diacylglycerol; TAG, triacylglycerol; PMA, phorbol 12-myristate 13-acetate; HBSS, Hanks balanced salt solution; BSA, bovine serum albumin; TLC, thin layer chromatography; PKC, protein kinase C; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

MATERIALS AND METHODS

Media for cultures, antibiotics, lipid standards and most enzymes were obtained from Sigma Chemical Company (St Louis, Mo.). Collagenase type I was purchased from Worthington Biochemical (Freehold, N.J.). Solvents were reagent grade. Fetal calf and horse serum were obtained from Hyclone (Provo, Ut.). Bovine serum albumin was from U.S. Biochemical Corp (Cleveland, Ohio). ACTH 1–24 (Cortrosyn) was purchased from Organon (West Orange, N.J.). [2-^{3}H]glycerol was from ICN (Irvine, Calif.) and had specific radioactivity of 2 Ci/mmol.

Adrenal cell culture

Calf adrenal glands were obtained at a local slaughterhouse under sterile conditions and placed immediately in a Hanks balanced salt solution (HBSS), containing 100 units/ml penicillin, 0.1 mg/ml streptomycin or 0.05 mg/ml gentamycin and 0.25 mg/ml amphotericin B, and taken to the laboratory. All further operations were then carried out in a laminar flow hood using sterile techniques. Adrenals were freed of fat and the outer 500 μ was sliced off with a Stadie-Riggs microtome. Slices were washed in HBSS and suspended in a modified Ham F-12 medium (142 mM Na^+, 1.58 mM $CaCl_2$, 2.94 mM KCl and 25 mM HEPES) containing 3.2 mg/ml collagenase, 0.4 mg/ml deoxyribonuclease I, 1 mg/ml protease IX, 0.5% BSA and the antibiotic mixture, and incubated at 37°C for 30 min. After incubation, the suspension was aspirated repeatedly with a 60 ml plastic syringe through latex tubing, and incubated for an additional 30 min. After this incubation, the suspension was aspirated 5 times as before, filtered through nylon mesh (210 μ), and the filtrate was saved. Cells were collected from combined filtrates by centrifugation (800 rpm × 8 min), washed twice with Ham F-12 medium containing 0.5% BSA, penicillin, streptomycin and amphotericin B, and finally resuspended in Ham F-12 medium containing 2.08 mM $CaCl_2$, 4.94 mM KCl, 10% mixed fetal calf + horse serum (1:4), antibiotic mixture, and an antioxidant mixture (4.5 μM Metyrapone, 45 nM selenous acid, 90 μM butylated hydroxyanisole and 0.5% dimethylsulfoxide) as described by Crivello *et al.*[15]. The cell suspension was then filtered through nylon mesh (80 μ), and, after counting the cells, aliquots (1.5×10^6 cells/2.5 ml medium) of this suspension were placed in wells of plastic multiwell culture plates (Costar, Cambridge, Mass) and incubated at 37°C under air with 5% CO_2.

Chronic and acute labeling experiments

After 24 h, media were removed, the attached cells were washed twice with HBSS. Ham F-12 medium (supplemented as before) was added, the cells were cultured over the next 3 days, in the presence or absence of 10 μCi of [2-^{3}H]glycerol. On the day of the experiment, the media were removed, and the cells were washed 3 times with HBSS, and fresh Ham F-12 medium was added (without isotope in 3-day prelabeling experiments). Cells were then equilibrated for 30 min at 37°C under air/5% CO_2, and ACTH or vehicle was added to each well for the times indicated. In acute labeling experiments, 20 μCi of [^{3}H]glycerol was added simultaneously with ACTH in 1 ml of medium or in medium alone (controls) and incubations were continued for the times indicated.

Incubations were stopped by placing plates on ice, aspirating the medium, and rapidly washing cells 3 times with ice-cold HBSS. 1 ml of ice-cold HBSS was added, and cells were scraped with a rubber policeman and transferred to tubes with two 0.5 ml washes. Cells were spun down (1500 rpm × 10 min at 4°C), washed once with ice-cold HBSS, suspended in 2 ml of the same medium and sonicated twice, using 12 pulses at 60% of the output control. After saving an aliquot for protein determination (approximately 0.5 mg per well), the homogenate was extracted with 7 vol of a mixture of $CHCl_3$:CH_3OH:0.1 N HCl (75:25:2) (extraction solvent). Organic extracts were washed 3 times with 2 ml of 0.01 N HCl, evaporated under nitrogen at room temperature, resuspended in extraction solvent, and examined for labeling of monoacylglycerol (MAG), DAG, triacylglycerol (TAG), PA, PI and PC + PE by thin layer chromatography as described previously [16–18]. DAG mass was measured by the DAG kinase method of Preiss *et al.*[19]. Inositol-phosphates in the aqueous phase were determined by chromatography on Dowex-1 columns (formate form, Biorad) as described previously [20–22].

Proteins were determined according to Bradford's method [23]. Aldosterone was measured in media by using direct radioimmunoassay [24].

Measurement of immunoreactive protein kinase C

Protein kinase C was purified from rat brain by successive column chromatography on DEAE-Cellulose and phenylsepharose CL-4B, followed by SDS–polyacrylamide gel electrophoresis (PAGE). The purified 80 kDa enzyme was localized by autoradiography following Ca^{2+}/phospholipid-dependent autophosphorylation, excised from the gel, mixed with Freund's adjuvant and used to immunize New Zealand white female rabbits. A polyclonal antiserum was obtained which recognized Types I, II and III rat brain protein kinase C (A more complete description has been published elsewhere [25]).

After treating cell cultures with 10 nM ACTH or vehicle (controls), cells were washed, centrifuged and homogenized as above except that the homogenization buffer contained 10 mM Tris-HCl buffer (pH 7.2), 25 mM sucrose, 10 mM EGTA, 25 mM $MgCl_2$, 2 mM PMSF and 10 μg/ml leupeptin. Cytosol and membrane fractions were obtained by centrifugation of homogenates at 100,000 g × 30 min. Supernatant (cytosol) was saved and the pellet was resuspended in the same buffer which also contained 1% Triton X-100, and centrifuged again under the same conditions. The resultant supernatant was used as the membrane fraction. Equal amounts of cytosol and membrane proteins were diluted 5:4 in Laemmli sample buffer [26] and subjected to SDS–PAGE on 8% gels. Separated proteins were electrophoretically transferred to nitrocellulose and then incubated successively with 3% gelatin, diluted antiserum, and goat anti-rabbit horseradish peroxidase. Specific immunoreactive bands were visualized with 4-chloro-1-naphtol and H_2O_2. Immunoblots were photographed and scanned with a laser densitometer (LKB BROMMA model XL). Absorbance of the major band at 80 kDa was taken as immunoreactive protein kinase C.

Statistical evaluation of results

In all cases, results are expressed as the mean ± SE of the individual number of samples in a representative experiment, which was repeated at least three times, each with virtually the same results. Statistical comparisons were made by Student's *t*-test.

RESULTS

Functional competence of cultured adrenal cells

When cultured cells were incubated with ACTH for 1 h, aldosterone production increased from 1.02 ± 0.85 ng/mg protein in control cells to 12.67 ± 1.29, 18.34 ± 1.64 and 23.56 ± 2.27 ng/mg protein in cells treated with 1, 10 and 100 nM ACTH respectively. When the incubation time was increased from 1 to 3 h, 10 nM ACTH increased aldosterone production 15- and 30-fold, respectively.

3-day [^{3}H]glycerol-prelabeling experiments

In cells which had been pre-labeled for 3 days with [^{3}H]glycerol, ACTH provoked time-dependent (Fig. 1) and dose-dependent (Fig. 2) increases in the labeling of DAG and total phospholipids (PL). The increase in DAG labeling was readily apparent within 1 min of ACTH (10 nM) addition, and was maximal (approximately 3-fold greater than control) at 2 min (Fig. 1). Total phospholipid labeling, on the other hand, increased progressively over the 5 min ACTH-treatment period (Fig. 1). Labeling of TAG was increased moderately (approximately 50% greater than control) within 2 min, but MAG labeling was relatively low and was not affected significantly by ACTH.

The labeling of individual phospholipids, PA, PI and PC + PE, were also examined and found to be increased in a time-dependent (Fig. 3) and dose-dependent (Fig. 4) manner. Labelling of these phospholipids increased equally rapidly in response to ACTH treatment, but labeling of PC/PE was greater in magnitude.

Changes in diacylglycerol content

The ACTH-induced increase in [^{3}H]DAG after 3 days of [^{3}H]glycerol prelabeling suggested that ACTH increases DAG content. This was verified by directly measuring DAG mass (Table 1), which was found to increase nearly 4-fold after 2 min of treatment with 10 nM ACTH. Lower (1 nM) and higher (100 nM) doses of ACTH also provoked lesser and greater increases in DAG content, as compared to 10 nM ACTH.

Acute [^{3}H]glycerol-labeling experiments

When ACTH was added simultaneously with [^{3}H]glycerol, time-dependent (Fig. 5) and dose-

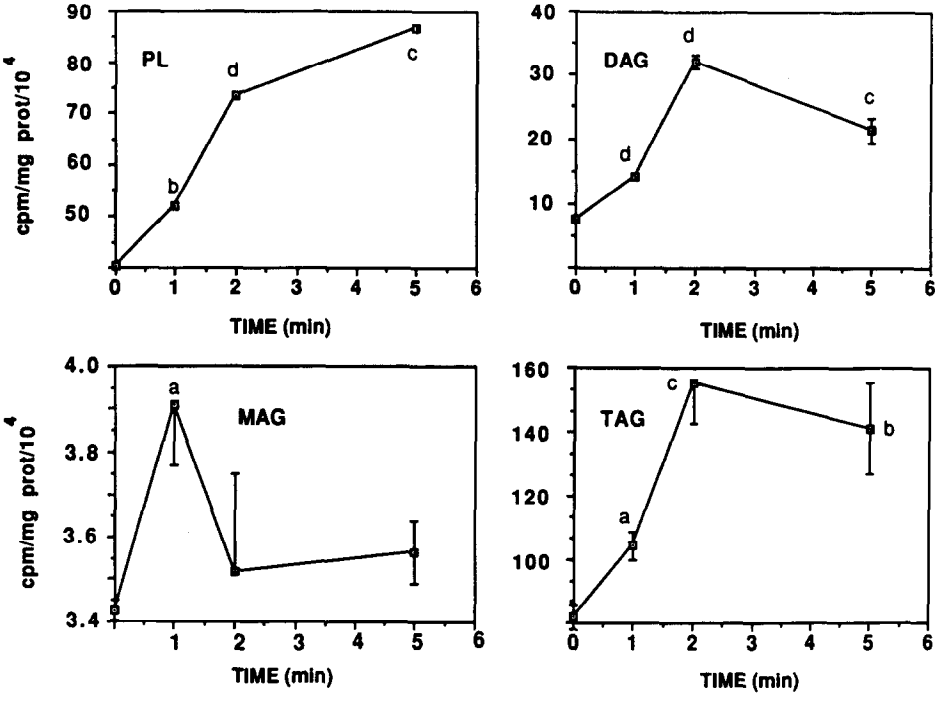

Fig. 1. Time-course of effects of 10 nM ACTH on labeling of total phospholipids (PL), monoacylglycerol (MAG), diacylglycerol (DAG) and triacylglycerol (TAG) after 3 days of prelabeling with [^{3}H]glycerol. After incubation, cells were harvested, washed, homogenized, extracted and lipids were analyzed by TLC. For other details, see the text. Values are the mean ± SE of 3 individual determinations of a representative experiment. Differences (ACTH versus control) were determined by Student's *t*-test: (a) $P < 0.05$; (b) $P < 0.02$; (c) $P < 0.01$ and (d) $P < 0.001$.

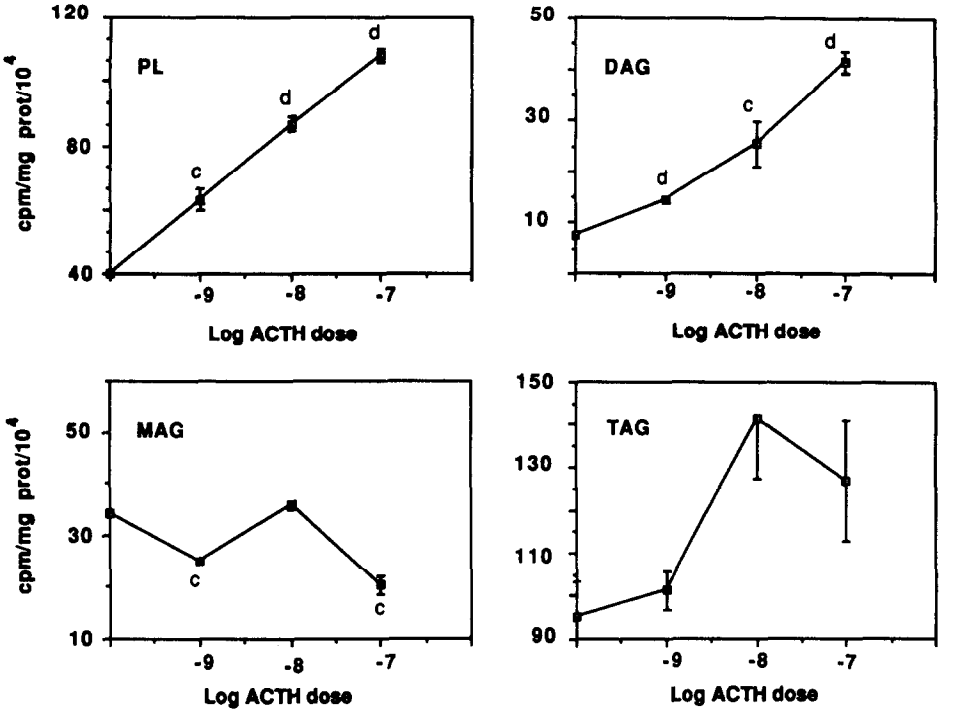

Fig. 2. Dose-related effects of ACTH on labeling of neutral lipids and total phospholipids after 3 days of prelabeling with [^{3}H]glycerol. Incubations ± ACTH were for 5 min. For abbreviations and other details see Fig. 1. Values are the mean ± SE of 3 individual determinations of a representative experiment. Differences (ACTH versus control) were determined by Student's t-test: (c) $P < 0.01$ and (d) $P < 0.001$.

dependent (Fig. 6) effects on labeling of DAG and PL were also observed and these were similar to those observed in 3-day pre-labeling experiments. No significant effects of ACTH were observed on MAG and TAG labeling, whereas labeling of total PL and DAG increased rapidly, nearly 2-fold within 2 min of ACTH addition. Of the phospholipids examined, labeling of PA, PI and PC + PE were all rapidly stimulated, but the largest ACTH-induced increases were seen in labeling of PA (Figs 7 and 8).

Changes in immunoreactive protein kinase C

The polyclonal antiserum recognized a major protein band from adrenal cells which migrated identically to that of highly-purified 80 kDa rat brain protein kinase C (data not shown).

Table 1. Effect of ACTH on DAG content in primary cultures of bovine adrenal glomerulosa cells

Incubation time (min)	ACTH concentration (nM)	DAG mass (nmol/mg protein)
0	0	22 ± 2 (5)
1	10	38 ± 3 (3)
2	10	88 ± 10 (5)
5	10	79 ± 6 (5)
2	1	32 ± 3 (3)
5	1	44 ± 5 (3)
2	100	100 ± 21 (3)
5	100	106 ± 15 (3)

Primary cells cultures were incubated with different ACTH concentrations for the indicated times. After incubations, media were aspirated, and cells were washed, harvested, homogenized and extracted with 7 volumes of extraction solvent. DAG mass was determined by the DAG kinase method (see Materials and Methods). Values are the mean ± SE of the number of determinations indicated in parentheses.

Cytosolic immunoreactive protein kinase C was almost 3-fold (2.76) greater than that of the membrane fraction in control cells. Figure 9 shows that within 2 min of ACTH treatment, cytosolic protein kinase C content decreased 50%, while membrane protein kinase C content increased 100%. Moreover, the total cytosolic plus membrane immunoreactive protein kinase C content was virtually unchanged by ACTH treatment at all incubation times employed (45.75 ± 1.11, mean ± SE). These results suggest that, in our primary cultures of calf adrenal glomerulosa cells, protein kinase C is stoichiometrically translocated from the cytosol to the membrane fraction in response to ACTH treatment. This apparent translocation is in agreement with, and complementary to, some previous reports of ACTH effects on protein kinase C activity [6, 13], but other groups reported increases in cytosolic protein kinase C enzymatic activity during ACTH treatment [11, 12]. The latter may be due to the fact that crude enzyme assays may reflect both enzyme content and activity, and ACTH-induced increases in diacylglycerol may cause disproportionate increases in enzyme activity. Obviously, our measurement of immunoreactive protein kinase C would only reflect enzyme content.

Similar translocation of immunoreactive protein kinase C was also produced by PMA (Fig. 9) a known activator of this enzyme [14].

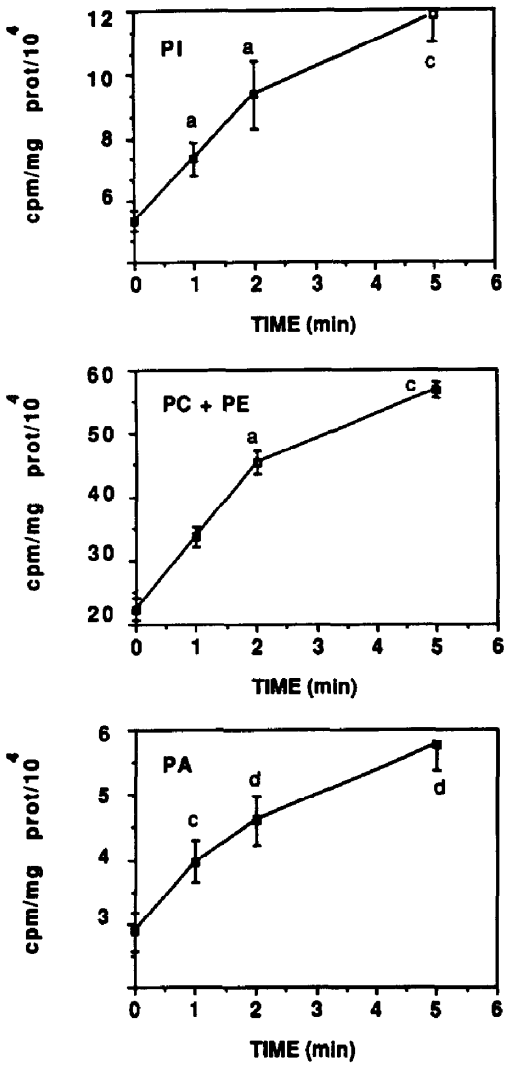

Fig. 3. Time-course of effects of 10 nM ACTH on labeling of phosphatidylinositol (PI), phosphatidylcholine plus phosphatidylethanolamine (PC + PE) and phosphatidic acid (PA) after 3 days of prelabeling with [^{3}H]glycerol. After incubations, phospholipids were analyzed by TLC as described in Materials and Methods section. Values are the mean ± SE of 3 individual determinations of a representative experiment. Differences (ACTH versus control) were determined by Student's t-test: (a) $P < 0.05$; (c) $P < 0.01$ and (d) $P < 0.001$.

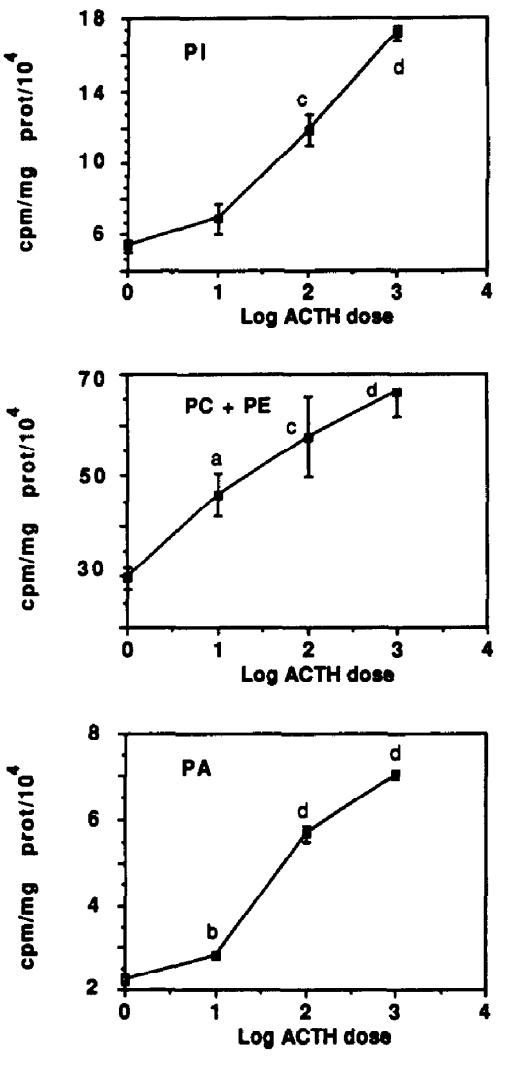

Fig. 4. Dose-related effects of ACTH on labeling of phospholipids after 3 days of prelabeling with [^{3}H]glycerol. Incubations ± ACTH were for 5 min. For abbreviations and other details see Fig. 3. Values are the mean ± SE of 3 individual determinations of a representative experiment. Differences (ACTH versus control) were determined by Student's t-test: (a) $P < 0.05$; (b) $P < 0.02$; (c) $P < 0.01$ and (d) $P < 0.001$. Abscissa: 0 = control; 1, 2, 3 and 4 = 1, 10, 100 and 1000 nM ACTH respectively.

DISCUSSION

Primary cultures of calf adrenal glomerulosa cells appear to be well suited for studies of hormonal effects on lipid metabolism. The cultured cells were very responsive to physiological doses of ACTH, and, as described below, long-term prelabeling with isotopes avoided some of the pitfalls which are inherent in acute labeling experiments.

In cells which were prelabeled for 3 days with [^{3}H]glycerol, despite removing isotope from the medium and repetitive washing with isotope-free medium, addition of ACTH provoked large, rapid increases in labeling of total phospholipids (including PA, PI and PC + PE) and TAG, as well as DAG. These increases in labeling indicate that: (a) ACTH increases *de novo* PA synthesis, since all glycerolipids are obligatorily derived from PA and total glycerolipid labeling was markedly increased by ACTH; (b) ACTH effects cannot be explained by changes in [^{3}H]glycerol uptake; and (c) these 3-day-prelabeled cells must contain sufficient reserves of [^{3}H]glycerol and/or [^{3}H]glycerol-3-PO_4 that can be utilized for *de novo* PA synthesis during acute ACTH stimulation (Note—All radioactivity is lost from [2-^{3}H]glycerol if it is converted to dihydroxy-acetone-PO_4 or other glycolytic intermediates).

The findings in acute [^{3}H]glycerol-labeling exper-

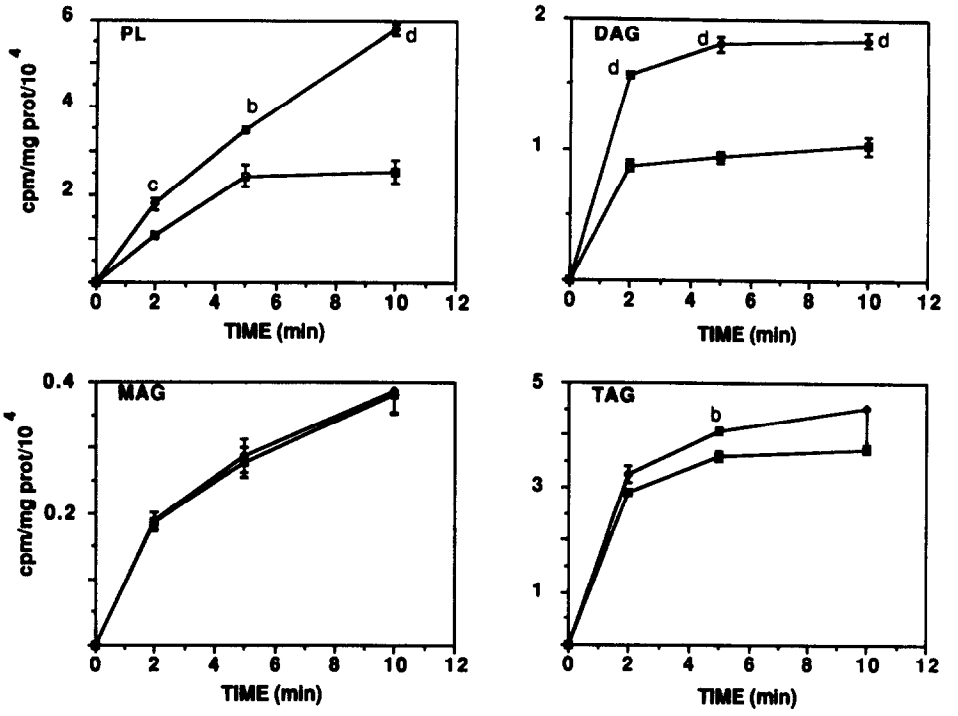

Fig. 5. Time-course of effects of 10 nM ACTH on labeling of total phospholipids and neutral lipids during acute labeling with [^{3}H]glycerol. Tracer was added to cells at zero time together with medium (controls) or ACTH. After incubations for the indicated times, cells were scraped, washed, homogenized and extracted. For abbreviations and other details see Fig. 1. Values are the mean ± SE of 3 individual determinations of a representative experiment. Differences (ACTH versus control) were determined by Student's t-test: (b) $P < 0.02$; (c) $P < 0.01$ and (d) $P < 0.001$.

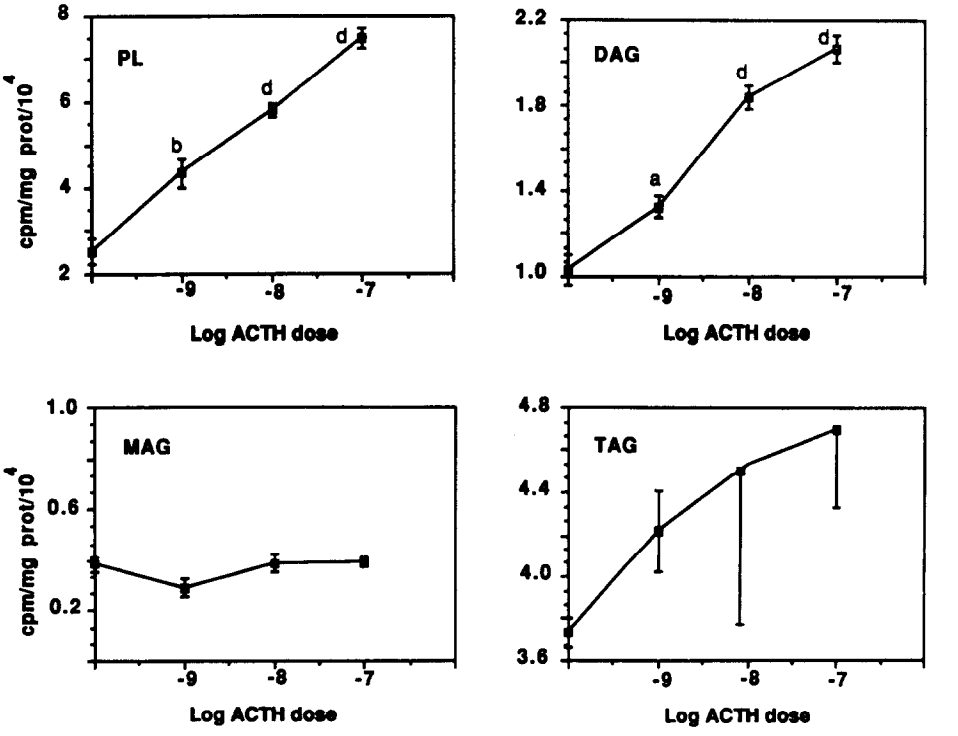

Fig. 6. Dose-related effects of ACTH on labeling of total phospholipids and neutral lipids during acute labeling with [^{3}H]glycerol. Tracer was added to cells simultaneously with ACTH. Incubations were for 5 min. For details see Fig. 2. Values are the mean ± SE of 3 individual determinations of a representative experiment. Differences (ACTH versus control) were determined by Student's t-test: (a) $P < 0.05$; (b) $P < 0.02$; (d) $P < 0.001$.

iments, in which isotope and ACTH were added simultaneously, provided further information that ACTH increased *de novo* PA synthesis, and that this contributed directly to increases in DAG. In these experiments, ACTH-induced increases in labeling of PA and DAG were more rapid and greater in magnitude than increases in labeling of PI and PC/PE. It is therefore unlikely that changes in [^{3}H]DAG labeling may be explained simply by increased hydrolysis of these phospholipids, although it is possible that the latter mechanism may contribute to increases in DAG mass.

The similar 3–4-fold ACTH-induced increases in DAG mass and [^{3}H]glycerol-labeling of DAG after 3-days of prelabeling is noteworthy and suggests that DAG was labeled to constant specific activity. In the acute [^{3}H]glycerol labeling experiments, on the other hand, it is surprising that the increase in the rate of labeling due to ACTH treatment was not as great as that observed in the 3-day prelabeling experiments; this suggests that other factors (e.g. labeling of the glycerol-3-PO_4 pool) may have been limiting in the acute labeling experiments.

As alluded to above, it is clear that ACTH must increase *de novo* PA synthesis, and furthermore, this must contribute to increases in DAG. The former seems clear, since (a) there were increases in total glycerolipid labeling in experiments using either labeling protocol (this indicates that there is not simply redistribution of pre-existing lipids); (b) in acute

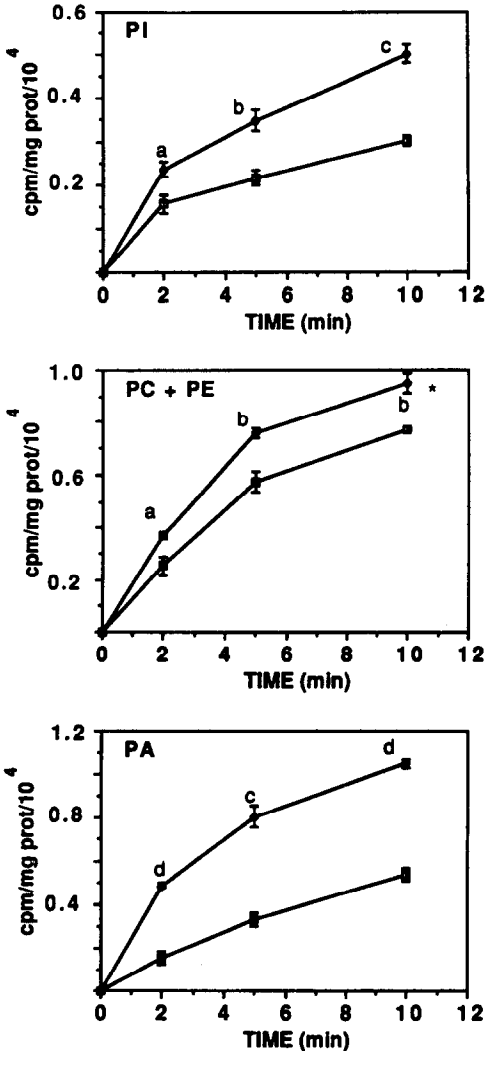

Fig. 7. Time-course of effects of 10 nM ACTH on labeling of phospholipids during acute labeling with [^{3}H]glycerol. Tracer was added to cells at zero time simultaneously with medium (controls) or ACTH. Incubations were for 5 min. For details see Figs 6 and 4. Values are the mean ± SE of 3 individual determinations of a representative experiment. Differences (ACTH versus control) were determined by Student's *t*-test: (a) $P < 0.05$; (b) $P < 0.02$; (c) $P < 0.01$ and (d) $P < 0.001$.

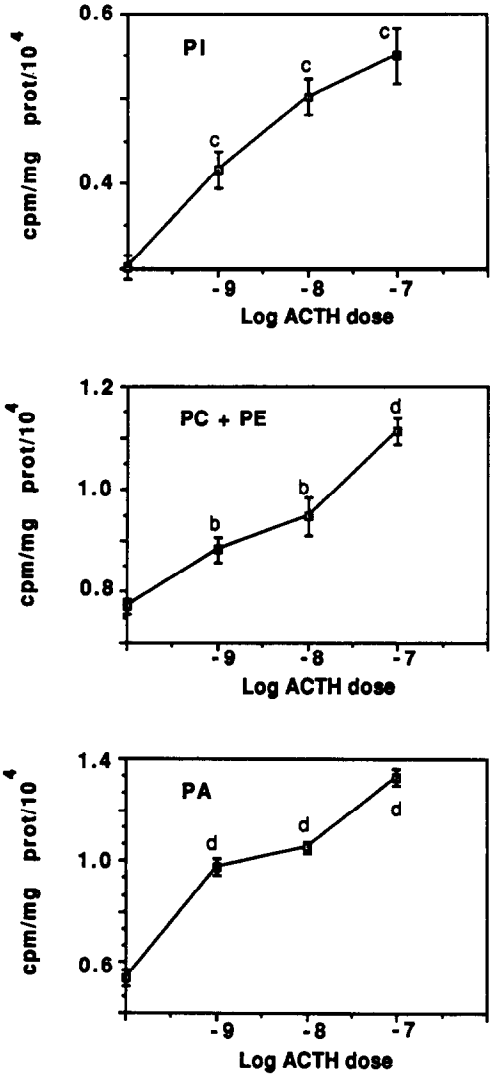

Fig. 8. Dose-related effects of ACTH on labeling of phospholipids during acute labeling with [^{3}H]glycerol. Tracer was added to cells at zero time with or without ACTH. Incubations were for 5 min. For details see Figs 6 and 4. Values are the mean ± SE of 3 individual determinations of a representative experiment. Differences (ACTH versus control) were determined by Student's *t*-test: (b) $P < 0.02$; (c) $P < 0.01$ and (d) $P < 0.001$.

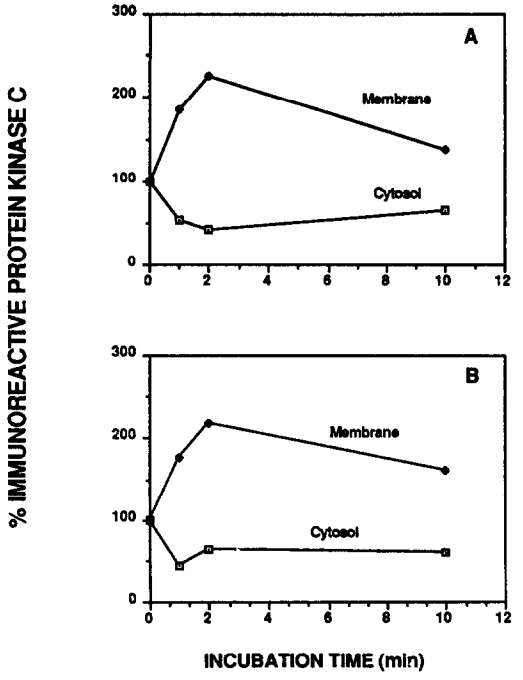

Fig. 9. Time-course of effects of 10 nM ACTH and 100 nM PMA on immunoreactive protein kinase C (PKC) in cytosol and membrane fraction. After incubations with ACTH (panel A) or PMA (panel B), cells were washed, harvested, homogenized and frationated in cytosol and membrane. Equal amounts of proteins were subjected to SDS–PAGE and separated proteins transferred to nitrocellulose and treated with diluted antiserum. Visualized bands were scanned and absorbance of the major protein at 80 kDa was graphed as immunoreactive protein kinase C.

labeling experiments PA was rapidly labeled and appeared to be the logical source of other lipids subsequently labeled. The fact that DAG must be derived at least partially from PA synthesized *de novo* also seems clear, since (a) DAG was very rapidly labeled (nearly as rapid as PA) in acute labeling experiments and could not be derived significant from sources other than PA (especially at early time points); (b) most newly synthesized PA is metabolized to TAG, PC and PE (rather than to inositol lipids), and DAG is an obligatory intermediate in these conversions.

Although ACTH has been reported to increase levels of a variety of phospholipids in the rat adrenal cortex [1–6] comparable increases have not been seen in incubations of bovine adrenocortical cells, including calf glomerulosa cells used presently, from the present results, however, it is clear that ACTH increases *de novo* synthesis of PA and other phospholipids, and it is clear that labeling techniques used presently are more sensitive indicators than changes in mass of phospholipids.

Although it seems clear that increases in *de novo* PA synthesis contributes to increases in DAG during ACTH action, the present results do not provide insight into the quantity of this DAG contribution and, of course, do not rule out hydrolysis of other lipids as the PI-glycan as important sources of DAG [27].

The fact that ACTH provoked the translocation of immunoreactive protein kinase C content from the cytosol to the membrane fraction is of interest for several reasons. First, this translocation is generally considered as a *sine qua non* for suggesting that protein kinase C has truly been activated during agonist action, and measurement of changes in enzyme activity, rather than enzyme content, may not provide definitive information on this point, since increases in DAG during hormone treatment may mask decreases in cytosolic enzyme content (see [25]). Secondly the translocation of protein kinase C provides further evidence that ACTH increases 1,2-*sn*-diacylglycerols rather than, or in addition to, 2,3-*sn*-diacylglycerols, since the latter do not activate protein kinase C [28, 29]. Increases in the later substances could theoretically contribute to observed increases in DAG, but not [2-^{3}H]glycerol-labeling of DAG.

In summary, ACTH provoked rapid and substantial increases in DAG in calf adrenal cells, and this increase appeared to be partially derived from *de novo* synthesis of PA, and was associated with translocation of immunoreactive protein kinase C, a mechanism thought to be indicative of protein kinase C activation [14]. The importance of hydrolysis of other lipids as sources of DAG and of the activation of protein kinase C during ACTH action remains to be evaluated.

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REFERENCES

1. Farese R. V., Sabir M. A. and Vandor S. L.: Adrenocorticotropin acutely increases adrenal polyphosphoinositides. *J. Biol. Chem.* **254** (1979) 6842–6844.
2. Farese R. V., Sabir M. A. and Larson R. E.: A23187 inhibits adrenal protein synthesis and the effects of adrenocorticotropin (ACTH) on steroidogenesis and phospholipid metabolism in rat adrenal cells *in vitro*: further evidence implicating phospholipids in the steroidogenic action of ACTH. *Endocrinology* **108** (1981) 1243–1246.
3. Farese R. V., Sabir M. A. and Larson R. E.: Comparison of changes in inositide and noninositide phospholipids during acute and prolonged adrenocorticotropin hormone treatment *in vivo*. *Biochemistry* **21** (1982) 3318–3321.
4. Igarashi Y. and Kimura T.: Adrenocorticotropin hormone-mediated changes in rat adrenal mitochondrial phopholipids. *J. Biol. Chem.* **259** (1984) 10745–10753.
5. Farese R. V., Larson R. E., Sabir M. A. and Gomez-Sanchez C. E.: Effects of angiotensin-II, K^+, adrenocorticotropin, serotonin, adenosine 3′,5′-monophosphate, guanosine 3′,5′ monophosphate, A23187 and EGTA on aldosterone synthesis and phospholipid metabolism in the rat adrenal zona glomerulosa. *Endocrinology* **113** (1983) 1377–1386.
6. Farese R. V., Fanjul L. F., de Ruiz Gallareta C. M., Davis J. S. and Cooper D. R.: ACTH increases diacylglycerol content and subcellular redistribution of

protein kinase C in the rat adrenal *in vivo*. *Life Sci.* **41** (1987) 2631–2637.
7. Farese R. V., Larson R. E. and Davis L. S.: Rapid effects of angiotensin-II on polyphosphoinositide metabolism in the rat adrenal glomerulosa. *Endocrinology* **114** (1984) 302–304.
8. Kojima I., Kojima K. and Rasmussen H.: Role of calcium and cAMP in the action of adrenocorticotropin on aldosterone secretion. *J. Biol. Chem.* **260** (1985) 4248–4256.
9. Enyedi P., Buki B., Musci I. and Spat A.: Polyphosphoinositide metabolism in adrenal glomerulosa cells. *Molec. Cell. Endocr.* **41** (1985) 105–112.
10. Nishizuka Y.: Studies and perspectives of protein kinase C. *Science* **233** (1986) 305–312.
11. Vilgrain I., Cochet C. and Chambaz E. M.: Hormonal regulation of a calcium-activated, phospholpid dependent protein kinase in bovine adrenal cortex. *J. Biol. Chem.* **259** (1984) 3403–3406.
12. Widmaier E. P. and Hall P. F.: Protein kinase C in adrenal cells: possible role in regulation of steroid synthesis. *Molec. Cell. Endocr.* **43** (1985) 181–188.
13. Ishizuka T., Miura K., Seigi N. and Nozawa Y.: Differential redistribution of protein kinase C in human aldosterenoma cells and adjacent cells stimulated with ACTH and angiotensin II. *Biochem. Biophys. Res. Commun.* **155** (1988) 643–649.
14. Castagna M., Takai Y., Kaibuchi K., Sano K., Kikkawa U. and Nishizuka Y.: Direct activation of calcium-activated, phospholipid dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* **257** (1982) 7847–7851.
15. Crivello J. F., Hornsby P. J. and Gill G. N.: Metyrapone and antioxidants are required to maintain aldosterone synthesis by cultured bovine adrenocortical zona glomerulosa cells. *Endocrinology* **111** (1982) 469–479.
16. Farese R. V., Konda T. S., Davis J. S., Standaert M. L., Pollet R. J. and Cooper D. R.: Insulin rapidly increases diacylglycerol by activating *de novo* phosphatidic synthesis. *Science* **236** (1987) 586–589.
17. Farese R. V., Larson R. E. and Sabir M. A.: Insulin acutely increases phospholipids in the phosphatidate-inositide cycle in rat adipose tissue. *J. Biol. Chem.* **257** (1982) 4042–4045.
18. Farese R. V., Barnes D. E., Davis J. S., Standaert M. L. and Pollet R. J.: Effects of insulin and protein synthesis inhibitors on phospholipid metabolism, diacylglycerol levels, and pyruvate dehydrogenase activity in BC3H-1 cultured myocytes. *J. Biol. Chem.* **259** (1984) 7094–7100.
19. Preiss J., Loomis C. R., Bishop W. R., Stein R., Niedel J. E. and Bell R. M.: Quantitative measurement of sn-1,2-diacylglycerols present in platelets, hepatocytes, and ras and sis-transformed normal rat kidney cells. *J. Biol. Chem.* **261** (1986) 8597–8600.
20. Berridge M. J., Dawson R. M. C., Downes C. P., Heslop J. P. and Irvine R. F.: Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem. J.* **212** (1983) 473–482.
21. Berridge M. J.: 5-Hydroxytryptamine stimulation of phosphatidylinositol hydrolysis and calcium signalling in the blowfly salivary gland. *Cell. Calcium* **3** (1982) 385–397.
22. Farese R. V., Davis J. S., Barnes D. E., Standaert M. L., Babischkin J. S., Hock R., Rosic N. K. and Pollet R. J.: The *de novo* phospholipid effect of insulin is associated with increases in diacylglycerol, but not inositol phosphates or cytosolic Ca^{2+}. *Biochem. J.* **231** (1985) 269–278.
23. Bradford M. M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* **72** (1976) 248–278.
24. Gomez-Sanchez C. E., Foecking M. F., Ferris M. W., Chavarri M. R., Uribe L. and Gomez-Sanchez E. P.: The production of monoclonal antibodies against aldosterone. *Steroids* **49** (1987) 581–587.
25. Acevedo-Duncan M., Cooper D. R., Standaert M. L. and Farese R. V.: Immunological evidence that insulin activates protein kinase C in BC3H-1 myocytes. *FEBS Lett* **244** (1989) 174–176.
26. Laemmli U. K.: Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature* **227** (1970) 680–685.
27. Cozza E. N., Vila M. C., Gomez-Sanchez C. E. and Farese R. V.: ACTH stimulates turnover of the phosphatidylinositol-glycan. *Biochem. Biophys. Res. Commun.* **157** (1988) 585–589.
28. Boni L. T. and Rando R. R.: The nature of protein kinase C activation by physically defined phospholipid vesicles and diacylglycerols. *J. Biol. Chem.* **260** (1985) 10819–10825.
29. Nomura H., Ase K., Sekiguchi K., Kikkawa U. and Nishizuka Y.: Stereospecificity of diacylglycerol for stimulus-response coupling in platelets. *Biochem. Biophys. Res. Commun.* **140** (1986) 1143–1151.