A POSSIBLE MECHANISM FOR THE STIMULATION OF KREBS CYCLE ACTIVITY BY ACTH IN ISOLATED RAT ADRENAL CELLS.

Ming-te Lin, Ajai Haksar and Fernand G. Peron

with the technical assistance of William F. Robidoux, Jr., and Susan R. Levy

Worcester Foundation for Experimental Biology

Shrewsbury, Massachusetts 01545

Received April 23, 1974

SUMMARY

Isolated rat adrenal cells, prepared by collagenase-trypsin treatment, were incubated in Krebs-Ringer phosphate buffer, pH 7.4, containing 0.5% BSA, 0.1% trypsin inhibitor and pyruvate-2-^{14}C. Krebs cycle activity was estimated by measuring the $^{14}CO_2$ evolved. ACTH caused a dose-dependent stimulation of $^{14}CO_2$ production in addition to the well known effect on steroidogenesis. When the steroidogenic effect of ACTH was inhibited with aminoglutethimide, the effect of the hormone on $^{14}CO_2$ production was also abolished. Aminoglutethimide, however, had no appreciable effect on the basal $^{14}CO_2$ production. Addition of 11-deoxycorticosterone, but not corticosterone, to the cells also stimulated $^{14}CO_2$ production. The data suggest that stimulation of Krebs cycle activity in the presence of ACTH may have been due to increased production of steroid precursor(s) of corticosterone which in turn would create an increased demand for reducing equivalents for their conversion into corticosterone.

INTRODUCTION

Earlier studies from our laboratory have indicated that both the formation of pyruvate and its utilization in the mitochondria may be essential for steroidogenesis in the rat adrenal cortex (1, 2). Besides the well known effect on steroidogenesis, ACTH and cyclic 3',5'-AMP have also been shown to stimulate the production of pyruvate and/or lactate from glucose (1-5). The effect of ACTH on the utilization of pyruvate in the adrenal cells, however, has not been studied. Recently, we have shown that pyruvate can efficiently supply carbon for the operation of the Krebs cycle in isolated rat adrenal cells (6). The Krebs cycle activity in these experiments was estimated by measuring the $^{14}CO_2$ evolved from pyruvate-2-^{14}C. In this report we describe

the stimulatory effect of ACTH on the Krebs cycle activity and suggest a possible mechanism for the observed effect.

MATERIALS AND METHODS

Chemicals. Trypsin and lima bean trypsin inhibitor were purchased from Worthington Biochemical Corp.; collagenase from Gallard Schlesinger Chemical Manufacturing Corp; bovine serum albumin (BSA), fraction V powder, fatty acid poor, from Miles Laboratories. Pyruvate-2-^{14}C was obtained from New England Nuclear Corp. and was diluted with non-radioactive pyruvate to get a specific activity of .05 μCi/μMole for use in the experiments.

Cell Suspensions. The technique for preparing adrenal cell suspensions has been described previously (2,7). In this study, however, there were two changes in the procedure (i) glucose was omitted from the buffer used in the preparation and (ii) for the final incubation, the cells were suspended in Krebs-Ringer phosphate buffer (KRP), pH 7.4, containing 5 mM sodium bicarbonate, 0.5% BSA and 0.15% trypsin inhibitor.

Incubations. Incubations were performed in 15 x 85 mm test tubes closed with serum stoppers which held plastic center wells (Kontes Glass Works, N. J.) for $^{14}CO_2$ collection. Each tube contained 1 ml of cell suspension, test substances dissolved in KRP and the requisite volume of KRP to make the final volume 1.5 ml. All tubes were gassed with 95% O_2:5% CO_2 before closing. Incubation was then carried out in a Dubnoff metabolic incubator with shaking at 37°C for 2 hours.

Measurement of $^{14}CO_2$. At the end of the incubation, 0.2 ml of a methoxyethanol: ethanolamine (2:1) solution (8) was injected through the serum stopper into the center well and 0.5 ml of 1 N HCl was injected into the cell suspension. Shaking was then continued for one hour to collect $^{14}CO_2$. The contents of the center well were transferred to counting vials and after addition of 10 ml of a counting fluid (84 ml liquifluor + 200 ml ethanol + 716 ml toluene) radioactivity was measured in a Packard liquid scintillation counter, model 3320, with a counting efficiency of 63%.

Corticosterone was measured by the method of Silber, et al. (9).

Each experiment was carried out in triplicate and averaged values are reported in the paper.

RESULTS AND DISCUSSION

ACTH produced a dose-dependent stimulation of corticosterone production from endogenous precursors as well as Krebs cycle activity which was determined by measuring the evolution of $^{14}CO_2$ from pyruvate-2-^{14}C (Figure 1). Maximum

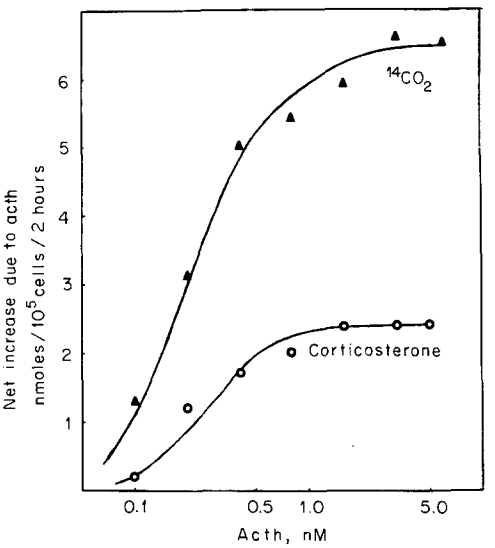

Fig. 1. Effect of ACTH on the production of $^{14}CO_2$ from Pyruvate-2-^{14}C and on the Synthesis of Corticosterone from Endogenous Precursors. Concentration of pyruvate-2-^{14}C was 5 mM. Basal values in the absence of ACTH were: corticosterone, negligible (< .05 nMoles); $^{14}CO_2$, 17.2 nMoles.

stimulation of $^{14}CO_2$ production, obtained with 1-2 x 10^{-9}M ACTH, was consistently 30-40% over the basal production in all experiments. A similar effect on $^{14}CO_2$ production was also observed with dibutyryl cyclic AMP; maximum stimulation being observed with 0.5-1.0 mM DBCAMP.

Figure 2 shows that ACTH increased the Krebs cycle activity at all concentrations of pyruvate tested. Maximum activity, both in the presence and absence of ACTH was obtained at the same concentration of pyruvate (1.5 mM).

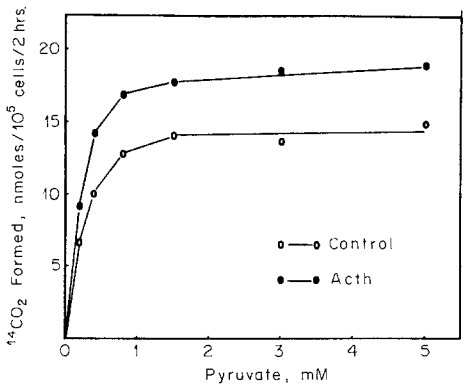

Fig. 2. Effect of ACTH (5 nM) on the Production of $^{14}CO_2$ at Different Concentrations of Pyruvate-2-^{14}C.

The stimulation of Krebs cycle activity by ACTH may be (i) due to a direct activation of some Krebs cycle enzyme(s) by cyclic AMP, the intracellular mediator of ACTH action on steroidogenesis (10-12) or (ii) it may be a secondary effect dependent on the increase in steroidogenesis. To distinguish between the two possibilities, experiments were carried out with aminoglutethimide which is known to inhibit the conversion of cholesterol to pregnenolone in several steroidogenic tissues (13-16). Table 1 shows that aminoglutethimide inhibited the ACTH-stimulated corticosterone as well as $^{14}CO_2$ production. The inhibitor, however, had almost no effect on the basal $^{14}CO_2$ production from pyruvate-2-^{14}C.

Because ACTH stimulates steroidogenesis in the adrenal cells, it is reasonable to expect that in the presence of ACTH there would be an increased utilization and, consequently, increased demand for replenishment of intramitochondrial NADPH, which is required for hydroxylation involved in the conversion of cholesterol to pregnenolone (17) as well as hydroxylations at 11β- and 18-positions on the steroid nucleus (18-20). Since our earlier studies have indicated that operation of the Krebs cycle may be essential for the generation of intramitochondrial NADPH required for 11β-hydroxylation of DOC (6), it is possible that in the presence of ACTH, the Krebs cycle activity was stimulated by the increased demand for NADPH in the mitochondria. This is supported by

Table 1. Effect of Aminoglutethimide on Krebs Cycle Activity and Corticosterone Formation

Amino-Glutethimide μM	Corticosterone Formed in the Presence of ACTH nMoles/10^5 cells/2 hrs	$^{14}CO_2$ Formed from Pyruvate-2-^{14}C nMoles/10^5 cells/2 hrs	
		+ ACTH	- ACTH
0	4.2	22.9	16.8
50	2.3	18.8	16.2
100	1.0	18.3	15.3
200	0.2	16.2	16.0

Corticosterone formation in the absence of ACTH was negligible (< .05 nMoles/10^5 cells) in all groups. Concentration of pyruvate-2-^{14}C was 5 mM and ACTH 5 nM.

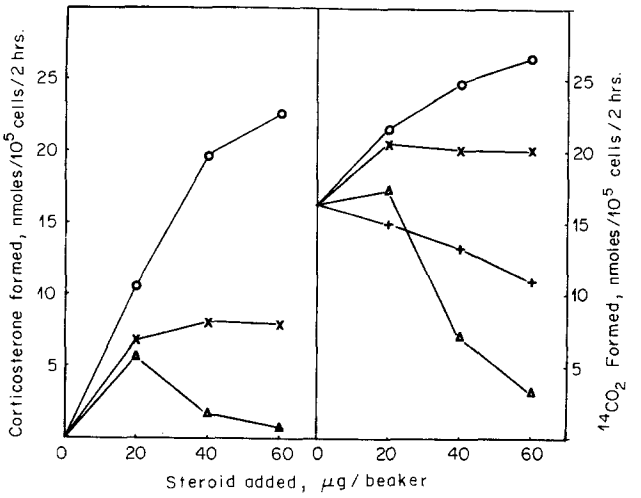

Fig. 3. Conversion of Pregnenolone, Progesterone and DOC into Corticosterone and the Effect of These Steroids and Corticosterone on $^{14}CO_2$ Production from Pyruvate-2-^{14}C. Each beaker contained 2 x 10^5 cells and 5 mM pyruvate-2-^{14}C in a final volume of 1.5 ml. Pregnenolone, X; progesterone, Δ; DOC, O; corticosterone, +.

the experiments reported in figure 3 where the efficacy of three steroid precursors to give corticosterone is correlated with their effect on the Krebs cycle activity. Both DOC and pregnenolone were converted to corticosterone by

the intact cells and both stimulated the Krebs cycle activity. It is interesting to note that DOC, which was converted to corticosterone more efficiently than pregnenolone, also stimulated the Krebs cycle activity to a greater extent. Progesterone, was found to inhibit the Krebs cycle activity at 40 and 60 µg levels and was a poor precursor of corticosterone at these levels. The findings with progesterone may be related to the reported inhibitory effect of this steroid on NADH oxidation and, consequently, ATP production in the mitochondria (21,22). Corticosterone, the end product of steroidogenesis in these cells, was also found to inhibit the Krebs cycle activity.

In conclusion, the data suggest that the increase in Krebs cycle activity by ACTH may be an adaptation mechanism devised in the adrenal cells and probably reflects an adjustment to increased demands for intramitochondrial reducing equivalents required for the hydroxylations involved in corticosterone production.

ACKNOWLEDGMENTS

This work was supported by grants GB-36246 from TheNational Science Foundation and AM-04899 and AM-05564 from the National Institutes of Health.

REFERENCES

1. Tsang, C.P.W., and Péron, F.G. Steroids 17, 453 (1971).
2. Haksar, A., and Péron, F. G. J. Steroid Biochem. 3, 847 (1972).
3. Kowal, J. Trans. N.Y. Acad. Sci. 31, 359 (1969).
4. Bell, J., Brooker, G., and Harding, B.W. Biochem. Biophys. Res. Comm. 44, 938 (1970).
5. Bartová, A., and Birmingham, M.K. Endocrinology 88, 845 (1971).
6. Lin, M.T., Haksar, A., and Péron, F.G. (Submitted for publication).
7. Haksar, A., Baniukiewicz, S., and Péron, F.G. Biochem. Biophys. Res. Comm. 52, 959 (1973).
8. Katz, J., Landau, B.R., and Bartsch, G.E. J. Biol. Chem. 241, 727 (1966).
9. Silber, R.H., Busch, R.D., and Oslapas, R. Clin. Chem. 4, 278 (1958).
10. Haynes, R.C., and Berthet, L. J. Biol. Chem. 224, 115 (1957).
11. Sutherland, E.W., Oye, E., and Butcher, R.W. Rec. Progr. Horm. Res. 21, 623 (1965).

12. Robison, G.A., Butcher, R.W., and Sutherland, E.W. Ann. Rev. Biochem. 37, 149 (1968).
13. Dexter, R.N., Fishman, L.M., Ney, R.L., and Liddle, G.W. J. Clin. Endocrinol. & Metab. 27, 473 (1967).
14. Kahnt, F.W., and Neher, R. Helv. Chim. Acta 49, 725 (1966).
15. Behrman, H.R., and Greep, R.O. Contraception 5, 229 (1971).
16. El Safoury, S., and Bartke, A. Steroids 23, 165 (1974).
17. Halkerston, I.D.K., Eicchorn, J., and Hechter, O. J. Biol. Chem. 236 374 (1961).
18. Hayano, M., Lindberg, M.C., Dorfman, R.I., Hancock, J.E.H., and Doering, W. von E. Arch. Biochem. Biophys. 59, 529 (1955).
19. Sweat, M.L., and Lipscomb, M.D. J. Am. Chem. Soc. 77, 5185 (1955).
20. Psychoyas, S., Tallan, H.H., and Greengard, P. J. Biol. Chem. 241, 2949 (1966).
21. Yielding, K.L., Tomkins, G.M., Munday, J.S., and Cowley, I.J. J. Biol. Chem. 235, 3413 (1960).
22. Varricchio, F., and Sanadi, D.R. Arch. Biochem. Biophys. 121, 187 (1967).