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STEROIDOGENESIS IN ADRENAL CELLS

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SUMMARY

It has been found that a potential exogenous steroid precursor such as cholesterol is not utilized by inhomogeneous rat adrenal cell suspensions, even under stimulation by ACTH or cAMP, unless exogenous NADPH or NADPH generation is provided. Furthermore, it has been shown that cAMP does not interfere with NADPH generation but stimulates NADPH utilization. A scheme is proposed for steroidogenesis in isolated rat adrenocortical cells considering precursor utilization and the various effects exerted by cAMP, NADPH and cycloheximide, respectively.

INTRODUCTION

As is well known, adrenal steroidogenesis requires besides oxygen at least three fundamental elements, i.e. (1) the steroid precursor which is believed to be free cholesterol, (2) a series of intra- and extramitochondrial enzymes, and (3) intra- and extramitochondrial cofactors such as NADPH and NAD.

In order to allow steroidogenesis to be stimulated by adrenocorticotrophic hormone (ACTH), these elements have to be highly compartmentalized as they are in the adrenocortical cell. Cyclic adenosine-3',5'-monophosphate (cAMP) seems to be the first intracellular mediator of the regulatory action of ACTH on the immediate steroidogenesis[1–4]. Though there is good evidence that cAMP action requires protein synthesis, the mechanism of this process is still unknown[5–9]. It seems reasonable to assume that cAMP acts directly or indirectly on the rate-limiting step of steroidogenesis, i.e. the conversion of cholesterol[10–14]. Using double labeled cholesterol as precursor and intact or ruptured mitochondria of bovine adrenocortical tissue, our kinetic results suggested that the transport of cholesterol from outside to inside the mitochondrial membrane is the proper rate-limiting step and not the subsequent enzymatic conversion of cholesterol to C_{21}-steroids[15]. It was also found that in contrast to some earlier suggestions[16, 17] no direct action of cAMP or its N^6-monobutyryl derivative (MB-cAMP) takes place on the rate-limiting step nor the subsequent enzymatic side chain split under the conditions examined. We concluded that the action of cAMP may be related only indirectly to this rate-limiting step and be involved either in the supply of the appropriate precursor or in its transport[15].

RESULTS AND DISCUSSION

In order to check the assumption made that free cholesterol is the appropriate precursor[13, 18–29], even under stimulation, we used a system which could be stimulated by ACTH or cAMP and where we could compare the endogenous precursor with various exogenous ones. When we incubated suspensions of isolated rat adrenocortical cells prepared according to standard procedures[30] with some modifications[31], the stimulation by ACTH, β^{1-24}-ACTH (Synacthen®), cAMP or MB-cAMP yielded up to 2–3 pg corticosterone per cell. This amount corresponds to about 10% of the total free endogenous cholesterol (20–30 pg/cell). When we added double labeled cholesterol (0·04 pg/cell), we assumed that it would equilibrate fairly quickly with the endogenous one expecting a similar conversion rate. However, we found that the exogenous cholesterol was not utilized within two hours to any measurable extent, not even under stimulation by ACTH or cAMP. This was in remarkable contrast to exogenous precursors such as pregnenolone and progesterone (0·26 pg/cell) or 20α-hydroxycholesterol[32] which were easily converted to corticosterone under unstable conditions. Exogenous cholesterol was neither utilized by intact cells in the presence of carrier proteins such as rat plasma or serum nor of electron donors such as isocitrate, nor by cells which had been broken up by various methods. It was only after addition of NADPH or NADPH generating systems to the cells, stimulated by cAMP or not, that the conversion of the cholesterol label was made possible to about 10–12%. At the same time, reducing equivalents of exogenous NADP were also able to stimulate steroidogenesis from endogenous precursors in this cell preparation.

Table 1. Relative utilization of precursors by isolated rat adrenal cells: the formation of corticosterone from endogenous precursor was measured by fluorometry[33]: ± stands for unstimulated steroidogenesis, whereas + + stands for about 30–50-fold stimulation: in case of exogenous double labeled cholesterol the side chain split was measured[15]: ○ = no split: the conversion of labeled pregnenolone or progesterone was followed by readioscanning of thin layer chromatograms

	Precursor		
	Endogenous	Exogenous Cholesterol	Pregnenolone/ progesterone
No stimulation	±	○	+
Isocitrate or malate (5×10^{-3} M)	±	○	+
Rat serum/plasma	±	○	+
Synthet. porcine ACTH or β^{1-24}-ACTH (Synacthen®) 10^{-10} M (±ATP 10^{-3} M)	+ +	○	+
+Cycloheximide 10^{-4} M	±	○	+
MB-cAMP 10^{-3} M (±ATP 10^{-3} M)	+ +	○	+
+Cycloheximide 10^{-4} M	±	○	+
+Isocitrate 5×10^{-3} M	+ +	○	+
NADPH 10^{-4}–10^{-3} M or NADPH-generating system	+ +	+	+
+ATP 10^{-3} M	+	+	+
+Cycloheximide 10^{-4} M	+ +	+	+

Table 1 gives a comparative survey of the relative utilization of precursors by isolated rat adrenal cells (100,000 cells/ml) as studied under various conditions.

Regarding the different utilization of endogenous precursor and exogenous cholesterol the following possibilities may be considered: (1) Presence of at least two different cell populations, (2) non-equivalence of the endogenous precursor to cholesterol.

(1) If we assume that we are dealing with a mixture of cell populations, the following possibilities, as shown in Table 2, may be envisaged. If a mixture of I and II with the same endogenous precursor would prevail, the stimulation of steroidogenesis by cAMP and NADPH should be strictly additive, even at high concentrations. From a technical point of view it has to be mentioned that the different batches of cell suspensions differ in absolute and relative sensitivity versus cAMP and NADPH generation from day to day, and contain no doubt a variable percentage of damaged cells which may increase after a 2 h incubation period.*

Nevertheless, it could clearly be demonstrated that at high concentrations after a 2 h incubation period additivity of steroid formation from endogenous precursors is no longer obtained (Fig. 1), whereas additivity is often seen at medium or low concentrations.

More interestingly, within a medium concentration range and shorter periods of incubation, synergic effects of cAMP and cofactor on the steroidogenesis from endogenous precursor were observed. This seems to eliminate a mixture of cell types I and II only.

Table 2. Hypothetical adrenocortical cell types with different responsiveness versus stimulation or inhibition of the conversion of endogenous precursor and exogenous cholesterol respectively

Cell type	Precursor	Stimulation by ACTH or cAMP	Stimulation by NADPH	Inhibition by Cycloheximide
I	endogenous	+	−	+
	exogenous cholesterol	−	−	−
II (e.g. damaged cells)	endogenous	−	+	−
	exogenous cholesterol	−	+	−
III	endogenous	+	+	+
	exogenous cholesterol	−	−	−

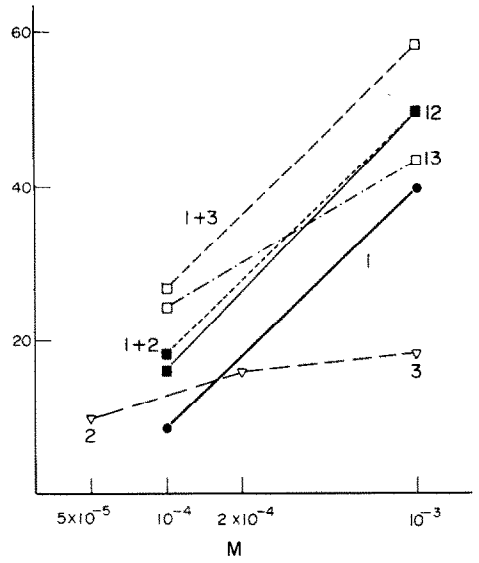

Fig. 1. Additive and non-additive effects of MB-cAMP and NADPH on steroidogenesis from endogenous precursor of isolated rat adrenocortical cells, incubation of 100,000 cells/ml KRBGA for 120 min at 37°C in 5% CO_2 and 95% oxygen. The amount of corticosterone is indicated on the ordinate as arbitrary fluorimeter units.

1: MB-cAMP
2–3: NADPH
12: MB-cAMP + 5×10^{-5} M NADPH, experimental curve
1 + 2: Additive curve, calculated
13: MB-cAMP + 10^{-3} M NADPH, experimental curve
1 + 3: additive curve, calculated
KRBGA: Krebs-Ringer-Bicarbonate + 0·2% Glucose + 0·5% Bovine Serum Albumine Fr. V.

However, it indicates that we may deal mainly with cells of type III in mixture with a varying percentage of type I and/or II (Table 2); both additivity, non-additivity and synergic effects would have to be expected; cells of type III may be slightly damaged in order to be permeable to NADPH but sufficiently intact in order to be stimulated by cAMP.

It seems worthwhile to dwell on the synergic effects of cAMP and NADPH-generation as seen in isolated cells since the early hypotheses of ACTH action proposed that cAMP acts by increasing the rate of NADPH-generation. As is well known, Haynes and co-workers[1] suggested that ACTH increases intracellular cAMP which could activate phosphorylase for an increase in glucose-6-phosphate (G-6-P) from glycogen: the increased supply of G-6-P would then lead to an increased rate of cytoplasmic NADPH generation. Subsequently, doubts have been expressed by many workers [cf. 34] as to the significance of phosphorylase activation for the regulation of steroidogenesis. An alternative hypothesis by McKerns[35] proposed that cAMP might activate directly the G-6-P dehydrogenase, another by Greenberg and Glick[36]

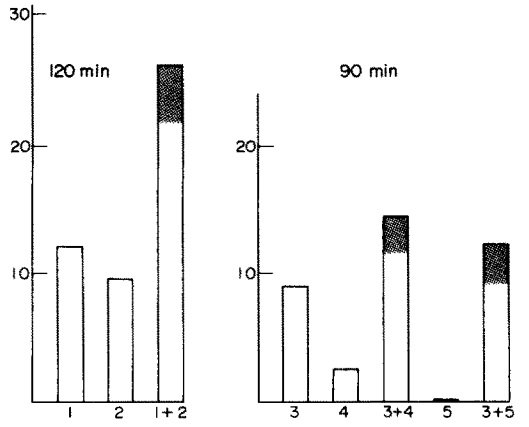

Fig. 2. Synergic effects of MB-cAMP and NADPH generation on isolated rat adrenal cell steroidogenesis.

1: 10^{-4} M MB-cAMP
2: 10^{-3} M NADP + 3×10^{-3} M glucose-6-phosphate
1 + 2: mixture
3: 3×10^{-4} M MB-cAMP
4: 3×10^{-4} M NADP + 2×10^{-3} M DL-Isocitrate
3 + 4: mixture
5: 2×10^{-3} M DL-Isocitrate
3 + 5: mixture

Synergic effects are represented as dotted areas. For other details cf. Fig. 1.

suggested that ACTH acts by activation of 6-phosphogluconate dehydrogenase in supplying more NADPH. A possible role for transhydrogenases was also implicated[37]. Working with isolated rat adrenal cells we have found that neither of these electron donors nor NADP alone gave rise to any appreciable conversion of the endogenous precursor to corticosterone.

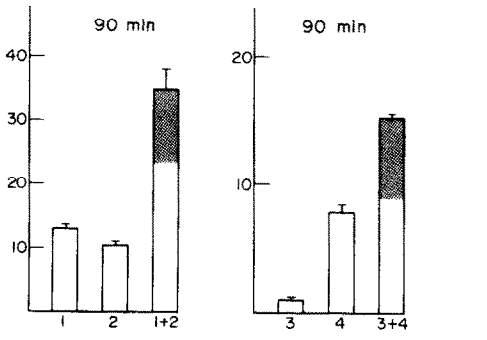

Fig. 3. Synergic effects of MB-cAMP and NADPH-generation on isolated rat adrenal cell steroidogenesis.

1: 3×10^{-3} M MB-cAMP
2: 2×10^{-3} M NADP + 2×10^{-3} M NADH
1 + 2: mixture
3: 10^{-4} M MB-cAMP
4: 5×10^{-4} M NADP + 5×10^{-3} M 6-phosphogluconate
3 + 4: mixture

Synergic effects are represented as dotted areas. For other details cf. Fig. 1. Experiments in duplicate ± S.D.

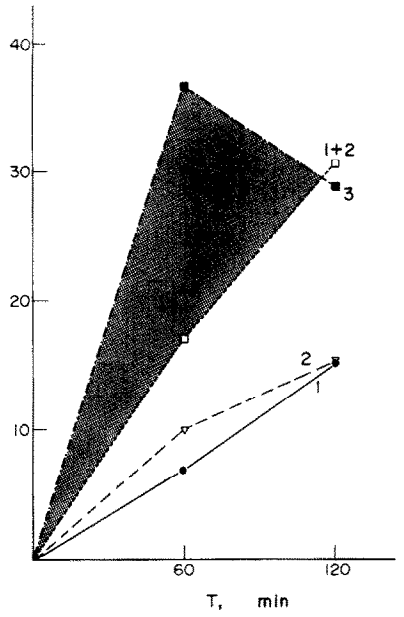

Fig. 4. Synergic effect of MB-cAMP and NADPH on steroidogenesis in isolated rat adrenal cells (batch a).
1: 10^{-3} M MB-cAMP
2: 10^{-4} M NADPH
3: 1 and 2 together, experimental curve
1 + 2: additive curve from 1 and 2, calculated
The dotted area indicates the range of synergism. For other details cf. Fig. 1.

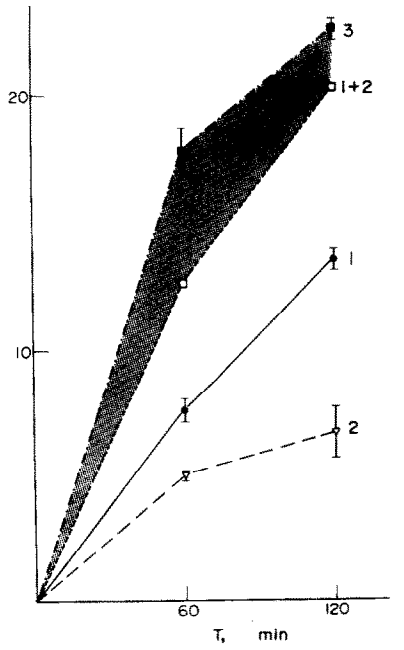

Fig. 5. Synergic effect of MB-cAMP and NADPH on steroidogenesis in isolated adrenal cells (batch b); for other details cf. Figs. 1 and 4. Experiments in triplicate ± S.D.

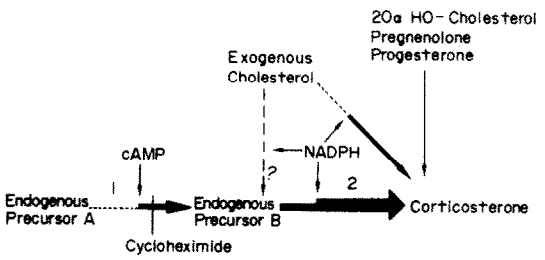

Fig. 6. Proposed interaction of endogenous and exogenous precursors and of cAMP and NADPH in isolated rat adrenal cell steroidogenesis.

However, if we used NADP in combination with NADH, G-6-P, 6-P-gluconate or isocitrate, a conversion similar to that obtained with NADPH or cAMP was usually seen. It was during these experiments that we have observed marked synergic effects of NADPH-generation and MB-cAMP as shown e.g. in Figs. 2 and 3. The magnitude of this activating effect of MB-cAMP proved to be dependent on relative cell sensitivity, concentration of both stimulants and time of incubation. This seemed first to support the hypotheses of ACTH-cAMP action mentioned above. However, more important was the subsequent finding that MB-cAMP had similar synergic effects on the stimulation by NADPH itself. In Figs. 4 and 5 this activation is demonstrated by two experiments showing some time dependence of the effect and some variations from one cell batch to the other. Our results lead to the conclusion that cAMP does not play a significant role in the regulation of NADPH-generation which seemed also unlikely from other considerations[38] and from the fact that cAMP did not stimulate utilization of the cholesterol label by isolated cells without added NADPH.

However, the observed synergic effects suggest that the utilization of NADPH itself is facilitated or favored in the course of the transformation of endogenous precursor to corticosterone if cAMP is present.

(2) Whereas it seems possible that the endogenous steroid precursor under stimulation is identical with cholesterol in the case of the hypothetical cells of type II, non-equivalence of endogenous precursor to cholesterol in cells of type I or III has to be considered.

In Fig. 6 a scheme is presented which takes into account that the actions of cAMP and NADPH on the endogenous precursor are in principle supporting two different steps which are sequential and interdependent. The first step is thought to be slower than the second one as indicated by the thickness of the arrow: such a scheme would allow additive, non-additive and synergic effects according to the disposition of the reactants. Step 1 comprises the cAMP-dependent conversion of endogenous precursor A to an intermediate precursor

B and can be inhibited by cycloheximide. This inhibition would imply that cAMP triggers the synthesis of regulatory protein involved either in carrying precursor A to the site of its conversion to precursor B or in producing the latter. In step 2 the intermediate precursor B is converted to corticosterone in a rate dependent on the concentration of reducing equivalents (NADPH); this step is not inhibited by cycloheximide.

It seems clear that precursors like 20α-hydroxycholesterol, pregnenolone or progesterone are post-B precursors and need no exogenous NADPH for conversion.

If precursor A would be identical with cholesterol, a cAMP-dependent carrier had to be postulated [cf. 7] which transports A to the site of conversion and precursor B would represent the corresponding enzyme-substrate complex. In this case MB-cAMP alone should be able to stimulate cholesterol conversion by intact cells at least to some extent. The present experimental data do not support this hypothesis and suggest rather non-equivalence of precursor A to cholesterol.

Since the characteristics of the conversion of cholesterol to C_{21}-steroids seem to correspond to step 2, the question then arises whether precursor B might be equivalent to cholesterol or not. If identity is proposed, one had to assume that the mixing of the cholesterol label with the endogenous cholesterol pool is not possible because in purified cell suspensions as of type I the label is not utilized even in the presence of NADPH[31].

In conclusion, our data suggest that cAMP does not interfere with any of the processes related to NADPH generation: it rather seems to amplify NADPH utilization by supplying an intermediate precursor at the site where C_{21}-steroids are generated. It will be the aim of future research to identify the endogenous precursors in adrenocortical cells stimulated by ACTH or cAMP on the one hand and on the other to pinpoint a regulatory protein the synthesis of which is under control of these stimulators.

* Addition in proof: Further purification of the cell suspension yielded a population behaving like cell type I[31].

mental design but using derivatives of cholesterol, such as

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DISCUSSION

Domínguez:

Doctor Neher, have you tried by any chance your experimental design but using derivatives of cholesterol, such as cholesterol sulfate, to see how this may operate?

Neher:

Not with isolated cells so far, but using isolated bovine adrenal mitochondria where we can convert exogenous cholesterol quite easily. When we add cold cholesterol sulfate we don't get any inhibition of the conversion of the hot label. From that experiment, at least, we concluded that cholesterol sulfate would have no function as a competitive precursor.

McKerns:

NADPH does not enter the intact cell very readily and I always have some reservations with cyclic AMP added into intact cells at high concentrations that there isn't a permeability effect, a non-physiological effect on the entry of the co-factor that is going to be utilized.

Neher:

We normally use monobutyryl cyclic AMP; morphologically the cells are more or less intact but how far NADPH could penetrate we don't really know. It is our feeling that NADPH could penetrate slightly damaged cells. There is obviously no penetration of NADPH of the mitochondrial membrane.

Müller:

It has been shown by Halkerston at the Worcester Foundation that NADPH can only enter broken cells. When added to quartered rat adrenals it stimulates the conversion of exogenous cholesterol to corticosterone out of proportion to its effect on endogenous corticosterone output. When I used quartered rat adrenals, exogenous cholesterol was hardly converted to corticosteroids and ACTH was ineffective. Then, I followed a suggestion by Koritz and added the labelled cholesterol with Tween 80. Under these conditions, ACTH had a stimulating effect on the incorporation of cholesterol into corticosterone which was more or less in proportion to its steroidogenic effect.

Neher:

Well, this cholesterol label was applied in the presence of some Tween 80 (10 μg/ml).

Härkönen:

I would like to know from which sources NADPH comes in the cells. Is it from the pentose shunt, isocitrate dehydrogenase or malic enzyme reactions? Do you have any idea about what is the main source of NADPH inside these cells?

Neher:

For the endogenous NADPH within the cell, I have no idea which is the prevailing source under the current condition. In our cell preparation we didn't do any experiment on that.

Kellie:

Was the exogenous cholesterol you added in the form of ester?

Neher:

It was free cholesterol.

Kellie:

Does it make any difference?

Neher:

Again, I have to come back to our experiments with mitochondria. If we used isolated bovine adrenal mitochondria, exogenous labelled free cholesterol is readily converted whereas labelled cholesterol acetate is not accepted at all.