THE EFFECT OF CALCIUM IONS ON THE METABOLISM OF EXOGENOUS CHOLESTEROL BY RAT ADRENAL MITOCHONDRIA

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1. Introduction

The short-term effect of adrenocorticotrophin (ACTH) in the adrenal gland is the stimulation of corticosteroid production. The rate-limiting step of adrenal steroidogenesis is the conversion of cholesterol to pregnenolone which occurs in the mitochondria of adrenocortical cells [1,2]. Since the trophic effect of ACTH with relation to increased corticosteroid production is prevented by prior treatment of the animal with cycloheximide the involvement of a putative protein factor in the regulation of the cholesterol desmolase system has been proposed [3]. There have also been reports which implicate calcium ions in the response of steroidogenic tissues to trophic hormone stimulation [4–7]. In these investigations [6,7] the influences of trophic hormones and calcium ions on the activity of the mitochondrial cholesterol desmolase towards endogenous cholesterol have been reported. Since in vivo adrenocortical mitochondria require a continuing supply of cholesterol to maintain steroid hormone production during trophic hormone stimulation (see Boyd et al. [8]), it was of importance to investigate the metabolism of extra-mitochondrial cholesterol by adrenal mitochondria and the effect of calcium ions on the process. Calcium ions were found to stimulate markedly pregnenolone synthesis from exogenous cholesterol. Calcium ions did not completely reverse the decreased cholesterol desmolase activity of adrenal mitochondria from cycloheximide-treated animals, suggesting that calcium ions are but one mode of regulating steroidogenesis.

2. Materials and methods

Female rats (180–200 g) of the Wistar strain obtained from the Small Animal Breeding Station, University of Edinburgh, were subjected to ether anaesthesia for 10 min, a stress facilitating increased blood ACTH levels [9], or were injected intraperitoneally with 10 mg cycloheximide (Koch-Light, Colnbrook) 10 min before sacrifice [10].

Mitochondria were prepared from the adrenal glands as previously described [10]. The mitochondria were washed once with 0.25 M sucrose and finally resuspended in the same medium at a protein concentration of about 4 mg/ml. Cholesterol side chain cleavage activity was determined as follows: incubations were carried out at 37°C in a buffer, pH 7.4, consisting of 250 mM sucrose, 20 mM KCl, 15 mM triethanolamine hydrochloride, 10 mM potassium phosphate, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% bovine serum albumin (Type F, Sigma) and 0.2 mM $NADP^+$, at a mitochondrial protein concentration of about 1 mg/ml and a total volume of 1 ml. Cyanoketone (2α-cyano-4,4,17α-trimethyl-17β-hydroxy-5-androsten-3-one), 6 μM, was included in the incubation to prevent the metabolism of pregnenolone to progesterone and further products [10]. Cholesterol was added in 10 μl acetone to a final concentration of 100 μM 3 min prior to initiation of the reaction with 10 mM DL-isocitrate. Enzymic activity was terminated by adding 0.2 ml aliquots to 4 ml of methanol. Steroids were extracted into an organic phase after addition of 4 ml chloroform and 2 ml of water. Pregnenolone concen-

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trations in the samples were estimated using a radioimmunoassay technique as described by Abraham et al. [11]. The pregnenolone antiserum, raised in sheep to a pregnenolone 20-albumin conjugate, was kindly supplied by Dr E. R. Simpson of the Department of Physics, Middlesex Hospital Medical School, London. The radioimmunoassays as well as recoveries of steroid from extractions were carried out using 7α-[^{3}H] pregnenolone of specific activity 20 Ci/mmole (New England Nuclear NET-093). Protein concentrations were estimated using the method of Lowry [12]. Mitochondrial cholesterol content was determined as described previously [10].

3. Results and discussion

Fig.1a and 1b shows the production of pregnenolone in the adrenal mitochondria from ether-stressed and cycloheximide-treated rats. It can be seen that in agreement with previous reports from this laboratory [6,10] the ether stress, which raises blood ACTH levels, caused the stimulation of pregnenolone production in these adrenal mitochondria in comparison of those from cycloheximide treated animals. On the addition of exogenous cholesterol to the mitochondria, from ether-stressed and cycloheximide-treated animals, it can be seen that pregnenolone production is stimulated. The pregnenolone production in the adrenal mitochondria from ether-stressed animals is much greater than those from cycloheximide-treated animals. Cycloheximide treatment of rats is thought to block the production of a factor important in the control of the metabolism of cholesterol by adrenal mitochondria [3]. Calcium ions have been reported to be important in controlling cholesterol metabolism [6]. It was therefore of interest to study the effects of calcium ions on the metabolism of added cholesterol

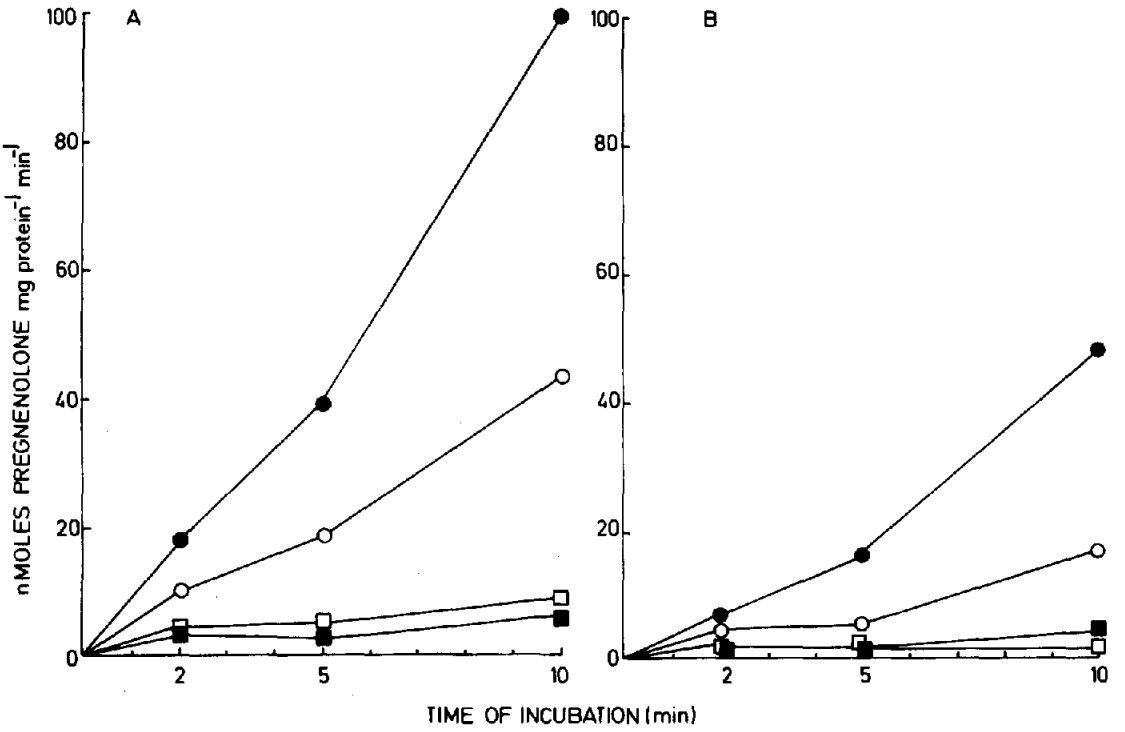

Fig.1. Pregnenolone production in adrenal mitochondria from ether-stressed and cycloheximide-treated rats. Incubations and pregnenolone determinations were carried out as described in the text: (□) Pregnenolone formation from endogenous substrate, (■) pregnenolone formed from endogenous substrate in the presence of 1 mM calcium chloride, (○) pregnenolone formation in the presence of 100 μM cholesterol, (●) pregnenolone formation in the presence of 100 μM cholesterol and 1 mM calcium chloride. (A) Adrenal mitochondria from ether-stressed rats. (B) Adrenal mitochondria from cycloheximide-treated rats.

by adrenal mitochondria. The results of such an experiment are shown in fig.1a and 1b. Calcium ions (1 mM) can be seen to have very little effect on the metabolism of endogenous cholesterol by the mitochondria of stressed or cycloheximide-treated animals. Addition of 100 μM cholesterol stimulated the production of pregnenolone in the mitochondria.

Addition of 1 mM calcium ions and 100 μM cholesterol together produced a two-fold rise in pregnenolone production in comparison to the addition of cholesterol alone. These concentrations of calcium and cholesterol were required for maximal steroid synthesis. Calcium ions had a stimulatory effect on the metabolism of exogenous cholesterol by adrenal mitochondria of cycloheximide treated animals although the rates of pregnenolone production are lower. Pregnenolone production occurs at a linear rate for at least 10 min in the presence of cholesterol or cholesterol plus calcium ions, in contrast to pregnenolone production from endogenous sterol which decreases after two min incubation. Thus the supply of cholesterol to adrenal mitochondria is very important in maintaining the rate of steroidogenesis. This is also true of the mitochondria of the corpus luteum and immature pig testis [13,14]. Trophic hormone action could provide this cholesterol by promoting the hydrolysis of cholesterol esters in cellular lipid droplets by cholesterol esterase [15]. The rapidly turning over factor [3] proposed to be involved in the control of adrenal steroidogenesis probably does not promote transport of cholesterol into the mitochondrion. Adrenal mitochondria isolated from ether-stressed rats contain less cholesterol (16.0 nmol/mg protein) compared to those obtained from cycloheximide-treated animals (27.0 nmol/mg protein) but the former mitochondria are capable of producing more pregnenolone in the presence of exogenous cholesterol (fig.1). Presence of cholesterol in mitochondria is therefore not sufficient to ensure high cholesterol side chain cleavage activity. Although calcium ions can influence markedly the rate of pregnenolone production, the metabolic block produced by cycloheximide administration cannot be reversed completely by addition of calcium ions at the optimal concentration. It is proposed therefore that the cycloheximide sensitive process is concerned with a direct activation of the cholesterol side-chain cleavage enzyme. Little evidence is at present available on the nature of this activation process, though the observation of activation of corpora luteal mitochondrial cytochrome *P*-450 by a protein kinase [17] would be a plausible mechanism. Calcium ions, on the other hand, appear to be involved in intramitochondrial translocation of cholesterol to enzyme binding sites as proposed recently by Simpson and William-Smith [16].

4. Conclusion

The results presented here suggest that calcium ions play a role in facilitating the metabolism of cholesterol taken up by rat adrenal mitochondria. This could be one important factor in the control of steroidogenesis by ACTH although other 'cycloheximide-sensitive' and as yet undefined, factors are required for maximal corticosteroid production.

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