THE EFFECT OF CALCIUM ON THE STIMULATION OF CORTICOSTERONE BIOSYNTHESIS BY DIBUTYRYL-C-AMP IN CULTURES OF ATCC CELL LINE Y-1*

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Summary

Monolayer cultures of mouse adrenal cortex tumors have been used to investigate the effect of calcium on the stimulation of steroidogenesis by dibutyryl-c-AMP. With the use of Ionophore A23187 as a tool, the present study strongly suggests that intracellular calcium is essential for dibutyryl-c-AMP-induced glucocorticoid synthesis.

Introduction

It is well established that the stimulation of glucocorticoid synthesis by ACTH requires Ca^{2+}. However, if one bypasses the hormone-receptor adenylcyclase system by using dibutyryl-c-AMP instead of ACTH, Ca^{2+} is no longer absolutely required. Instead, results from various laboratories showed that Ca^{2+} may have no effect on or may slightly stimulate (<100%) dibutyryl-c-AMP induced glucocorticoid synthesis (see Kowal et al. (3) and the references therein). This inconsistency exists not only in different laboratories, but has also been noted in our laboratory from one experiment to another. Since intracellular Ca^{2+} has been postulated to be required for many functions, (see discussion), we postulated that the experimental variations may be related in part to intracellular Ca^{2+} content. With the use of the Ca^{2+} ionophore A23187 (4) we have obtained results suggesting that intracellular Ca^{2+} is essential for dibutyryl-c-AMP-induced glucocorticoid synthesis.

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Materials and Methods

ACTH-responding adrenal cortex cell line isolated by Yasamura et. al. (5) was obtained from the American Type Culture Collection. Cells were grown in 30 ml. Falcon flasks in growth medium (F10 medium with 15% horse serum and 2.5% fetal calf serum) to log phase without ACTH. Prior to the experiment, the growth medium was removed and the cultures were incubated for 30 min. in KRHG medium (standard Kreb-Ringer solution without calcium, 20 mM Hepes buffer pH 7.4 [hydroxyethyl peperazine ethane sulfonic acid] and 0.2% glucose). This procedure facilitated the removal of preformed steroids and enabled the cells to adapt to the change of growth medium to buffered salt solutions used later in the experiment. As the experiment began, the flasks were divided into four groups. One group of four flasks serving as controls was incubated in KRHG with 5 mM Ca^{2+} added. A second group of eight flasks was treated for 8 min. at room temperature with STKG medium (0.15 M deionized sucrose, 5 mM Tris, pH 7.4, 0.5 mM KCl and 0.2% glucose). The third group of eight flasks was treated with a STKGE medium (STKG medium plus 0.1 mM EGTA). The fourth group of eight flasks was treated with STKGEA medium (STKGE medium plus 4 mM Ca^{2+} ionophore A_{23187}). After these 8 min. treatments, the media were removed and the cultures were washed once with KRHG medium. All the flasks were then incubated at 37° C for 2 hours in KRHG medium with the addition of ACTH, dibutyryl-c-AMP and/or Ca^{2+} (see results, table I). After incubation, the media were removed for corticosterone determination (6). The cells were digested with 1 N NaOH overnight at 37°C and the total protein content determined (7).

Results and Discussion

The results of a typical experiment are shown in Table I. Samples 1

Table I Effect of Calcium on the Stimulation of Steroidogenesis by dibutyryl-c-AMP

Addition to medium	control		STKG treated				STKGE treated				STKGEA treated			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
none	0.43		0.13				0.20				0.13			
1mM Ca^{2+}				0.26				0.24				0.24		
$2x10^{-4}$M dbcAMP					0.46				0.40				0.12	
Ca^{2+} + dbc AMP						0.59				0.56				0.91
20 m units/ml ACTH		2.40												
protein/flask	0.80	0.82	0.87	0.92	0.94	0.92	0.72	0.75	0.73	0.72	0.72	0.75	0.83	0.81
Ratio of Ca^{2} + dbc AMP stimulated output to dbc-AMP stimulated output				$\frac{\#6}{\#5} = 1.3$				$\frac{\#10}{\#9} = 1.4$				$\frac{\#14}{\#13} = 7.6$		

Note: 1. Data are the average of duplicates. The steroid output is expressed as μg corticosterone/mg protein. Standard deviation of the experimental results is 0.1.

2. Sample 1 and 2 have 5 mM Ca^{2+} with the KRGH medium.

3. dbc-AMP: dibutyryl-c-AMP

4. protein/flask: mg. of protein

and 2 show that the control cultures in Ca^{2+}-containing KRHG medium responded to the addition of ACTH by a six fold increase in steroid output. The basal output of corticosterone for the experimental groups were all lower (samples 3, 7, and 11) than normal (sample 1), presumably due to the STKG pretreatment of the cells. In both STKG and STKGE treated groups, the cultures responded to dibutyryl-c-AMP by a two to four fold increase in steroid output (compare samples 3 to 5 and 7 to 9). Ca^{2+} is not absolutely required, but may be slightly stimulatory (compare samples 5 to 6 and 9 to 10) or without effect (results of other unpublished experiments). In contrast, the ionophore treated cells did not respond to dibutyryl-c-AMP alone (compare sample 11 to 13). However, when both Ca^{2+} and dibutyryl-c-AMP were added, there was an approximately seven fold stimulation of corticosterone output (compare sample 11 to 14). The ratio of Ca^{2+} plus dibutyryl-c-AMP stimulated output over the lone dibutyryl-c-AMP stimulated output also clearly demonstrated that the calcium requirement became more pronounced after ionophore treatment (bottom row, Table 1). Presumably, with the ionophore rendering the cells leaky to Ca^{2+} and the EGTA chelating the Ca^{2+}, these cells were depleted of their intracellular Ca^{2+} and were unresponsive to dibutyryl-c-AMP unless Ca^{2+} was added. The intracellular Ca^{2+} is apparently "bound" because pre-treatment with the ionophore without the chelator EGTA did not result in a requirement for Ca^{2+} (unpublished results).

The importance of Ca^{2+} in many biological processes is well known (8). However, with the exception of muscular contraction, the mechanism of action of Ca^{2+} is poorly understood. At the subcellular level, Ca^{2+} is required for the maintenance of normal mitochondrial ultrastructure (9). At the cellular level, Ca^{2+} flux between intra and extracellular space has been postulated to play an important regulatory function (8, 10).

The results presented here are in accord with, but do not as yet prove, the postulate that the steroidogenic effect of dibutyryl-c-AMP is dependant on intracellular Ca^{2+}. We have previously reported that the adrenal cortex mitochondria contain a Ca^{2+}-activated malic enzyme and have postulated that this enzyme may play a key role in the supply of TPNH for mitochondrial sterol oxygenases (11). This would be one possible explanation for the requirement of intracellular Ca^{2+} for steroidogenesis. However, these treated cells are obviously defective in some unknown fashion as the absolute amounts of steroid secreted is somewhat reduced as it is stimulated by dibutyryl-c-AMP. It should also be pointed out that the depletion of intracellular Ca^{2+} by treatment with EGTA and the ionophore is inferred but not proven. Furthermore, the mechanism of c-AMP stimulation of steroidogenesis is complex and as yet poorly understood, but certainly involved translational control. The results presented here are the starting point towards further studies on the possible role(s) of intracellular Ca^{2+} in the adrenal cortex. Work is in progress towards the determination of intracellular Ca^{2+} content and the effect of Ca^{2+} addition on protein synthesis and on mitochondrial ultrastructure and malic enzyme activity.

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