COMPARISON OF THE Ca^{++} REQUIREMENT FOR THE STEROIDOGENIC EFFECT OF ACTH AND DIBUTYRYL CYCLIC AMP IN RAT ADRENAL CELL SUSPENSIONS

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ABSTRACT

Calcium requirement for ACTH and Dibutyryl cyclic AMP (DBCAMP) stimulation of steroidogenesis was compared in rat adrenal cell suspensions. In the absence of added calcium ACTH at low concentrations (< 1 mU/ml) was ineffective; however, the calcium requirement decreased when higher concentrations of ACTH were used. This was not the case with DBCAMP. At all levels of the nucleotide tested, the Ca^{++} requirement was about the same. When the cells were preincubated with EGTA, the Ca^{++} requirement became more pronounced for ACTH than for DBCAMP. The results indicate that the events before the formation of cyclic AMP show a greater dependence on Ca^{++} than the events following its formation.

INTRODUCTION

The following steps in the action of ACTH on steroidogenesis in the adrenal cortex have been well established: first, binding of ACTH to a receptor on the cell membrane (1,2); second, activation of adenyl cyclase (3,4) to increase the intracellular levels of cyclic AMP (4); and third, stimulation of cholesterol side chain cleavage by cyclic AMP (5,6). The mechanism for the third step is not known fully although synthesis of a labile protein has been suggested as obligatory (7,8). The requirement of Ca^{++} for the steroidogenic effect of ACTH has been known for many years (9,10); however, the exact nature of its participation in the chain of events involved in the ACTH response is not clear. Birmingham had shown that Ca^{++} is required for the third step i.e. steroidogenic action of cyclic AMP (11) and recently Farese has suggested that this Ca^{++} requirement may be at the protein synthesis level (12,13). Ca^{++} has also been found to regulate the second step i.e. activation of adenyl cyclase in membrane fractions obtained from adrenal homogenates (14,15,16). Finally several investigators have suggested that Ca^{++} may be required for the binding of ACTH to the cell membrane (17,18).

In this report we present data on the comparison of Ca^{++} dependence for the events before and after the formation of cyclic AMP.

METHODS

The technique of preparing cell suspensions was essentially that of Sayers et al. (19) with some changes. The following solutions were prepared in Ca^{++} free Krebs-Ringer bicarbonate buffer, pH 7.2, containing 2 mg/ml glucose (KRBG-Ca^{++}).

Solution A - 150 Units of collagenase (Worthington) per ml of KRBG-Ca^{++}.

Solution B - 2.5 mg of trypsin (Worthington) per ml of KRBG-Ca^{++}.

Solution C - 3.0 mg of lima bean trypsin inhibitor (Worthington) + 7.5 mg of bovine serum albumin (BSA; Pentex) per ml of KRBG-Ca^{++}.

Adrenals from 16-20 rats were cut into small pieces and placed in a flask containing 2 ml of Solution A. The flask was swirled gently for one minute, 18 ml of Solution B was added and two glass marbles introduced in the flask which was then incubated in a Dubnoff metabolic shaker (90-100 shakes/min.) for 20 minutes at 37° in an atmosphere of 95% O_2:5% CO_2. The flask was placed on ice to allow intact tissues to settle and the supernatant, containing isolated cells, was collected in another flask. This collagen - trypsin treatment was repeated 4-6 times more. The pooled supernatant was centrifuged at 100 xg for 40 minutes at 4°C and the sedimented cells were suspended in a known volume of Solution C.

Table 1 Effect of Ca^{++} on the Steroidogenic Response of Rat Adrenal Cells to ACTH and DBCAMP.

Exp.	Additions	Ca^{++} added in the medium, μM: 0	20	50	200	1000	B_{1000}/B_0
		Corticosterone formed, μg/beaker.					
1	ACTH 100 μU/ml	0	0	0	0	0.28	∞
	300 "	0	0	0.08	0.70	1.30	∞
	1 mU/ml	0.13	0.42	0.77	1.45	1.70	13.1
	3 "	0.76	0.90	1.30	1.75	2.05	2.7
	10 "	1.00	1.35	1.40	1.75	1.95	2.0
2	DBCAMP 25 μM	0.28	0.28	0.31	0.40	0.39	1.4
	100 "	1.16	1.34	1.51	1.59	1.68	1.4
	250 "	1.57	1.59	1.76	2.00	2.22	1.4
	500 "	1.68	1.74	1.96	2.12	2.28	1.4
	1000 "	1.53	1.65	1.80	2.08	2.23	1.4

Each beaker contained 170,000 cells in expt. 1 and 180,000 cells in expt. 2. B_{1000}/B_0 denotes the ratio between corticosterone formed in the presence of 1000 μM Ca^{++} and in the absence of Ca^{++}.

All incubations were performed in duplicate for 2 hrs. in a Dubnoff metabolic shaker at 37° in an atmosphere of 95% O_2:5% CO_2. Each beaker contained 1.0 ml of the cell suspension in Solution C, test substances dissolved in KRBG-Ca^{++} and an appropriate volume of KRBG-Ca^{++} to make the final volume 1.5 ml.

Corticosterone was measured by the fluorometric method of Silber et al. (20).

RESULTS AND DISCUSSION

In the preliminary experiments carried out in Ca^{++} free Krebs-Ringer bicarbonate buffer with varying Ca^{++} concentrations (10 μM to 10 mM) it was found that ACTH produced maximal effects on corticosterone synthesis at about 1-2 mM Ca^{++}. On the other hand maximal effects of DBCAMP were obtained at 0.2 - 0.5 mM Ca^{++}. At 2 mM Ca^{++} maximum stimulation of steroidogenesis was obtained with 1-2 mU/ml ACTH or 0.2 - 0.5 mM DBCAMP.

Table 1 shows that there was an absolute Ca^{++} requirement for the steroidogenic action of small amounts of ACTH (< 1 mU/ml). At higher levels of ACTH, however, the Ca^{++} requirement was diminished and the ratio of corticosterone produced at 1000 μm Ca^{++} to that produced in the absence of Ca^{++} (B_{1000}/B_0) was substantially decreased. This was not the case with DBCAMP; at all levels of the nucleotide tested the B_{1000}/B_0

Table 2 Calcium Dependence Compared at High Concentrations of ACTH and DBCAMP.

Additions	Ca^{++} added in the medium, μM		$\frac{B_{2000}}{B_0}$
	0	2000	
	Corticosterone formed, μg/beaker		
ACTH 1 mU/ml	0.39	2.07	5.3
10 "	1.27	2.17	1.7
25 "	1.51	2.07	1.4
50 "	1.47	2.11	1.4
DBCAMP 0.5 mM	1.53	2.05	1.3
1.0 "	1.51	2.16	1.4
2.5 "	1.63	2.13	1.3
5.0 "	1.57	2.02	1.3

Each beaker contained 180,000 cells. B_{2000}/B_0 denotes the ratio between corticosterone formed in the presence of 2000 μM Ca^{++} and in the absence of Ca^{++}.

Table 3 Calcium Dependence in Cells Preincubated in Ca^{++}-Free Media With or Without EGTA.

Additions	Preincubated without EGTA			Preincubated with EGTA		
	Incubation Ca^{++}, μM		$\frac{B_{2000}}{B_0}$	Incubation Ca^{++}, μM		$\frac{B_{2000}}{B_0}$
	0	2000		0	2000	
	Corticosterone formed, μg/beaker			Corticosterone formed, μg/beaker		
ACTH 1 mU/ml	0.17	1.55	9.1	0.11	1.50	13.6
10 "	1.08	1.48	1.4	0.70	1.48	2.1
20 "	1.14	1.48	1.3	0.84	1.43	1.7
DBCAMP 100 μM	0.95	1.26	1.3	0.75	1.08	1.4
500 μM	1.19	1.48	1.2	0.99	1.43	1.4

Cells were preincubated for 1 hour in Solution C with or without EGTA, washed with KRBG-Ca^{++} and then resuspended in Solution C for the final 2 hour incubation. Each beaker contained 125,000 cells.

ratio was about 1.4. Table 2 shows that with very large amounts of ACTH (25 or 50 times the maximally stimulating levels) the amounts of corticosterone produced at 0 and 2000 μM Ca^{++} were about the same as those produced with maximally stimulating concentrations of DBCAMP. As a result the ratio B_{2000}/B_0 for ACTH was almost the same as that for DBCAMP indicating that with these large amounts of ACTH only the reactions occurring after the formation of cyclic AMP show a requirement for Ca^{++}.

According to Birmingham et al. (11) and Farese (12) the Ca^{++} requirement for the action of ACTH and cyclic AMP in the quartered adrenals is about the same. However, they used high levels of ACTH in their experiments which might mimic the conditions in our experiments carried out with large amounts of ACTH. In addition, there might be substantially more endogenous Ca^{++} in the cells of adrenal sections used by these investigators than in the isolated cell suspension used by us. We have prepared the cells in a Ca^{++}-free medium which must have depleted the cells of at least some of the endogenous Ca^{++} and have shown that low to maximally stimulating levels of ACTH have an absolute requirement for Ca^{++} whereas DBCAMP does not.

Because high levels of ACTH showed a diminished Ca^{++} requirement it was pertinent to study the Ca^{++}requirement in cells after depleting the endogenous Ca^{++} with EGTA (Ethyleneglycol-bis (β-aminoethyl Ether)-N,N^1-tetraacetic acid). In the experiment reported in Table 3, cells were preincubated for one hour either in Solution C or Solution C containing 1 mM EGTA, washed once with KRBG-Ca^{++} and then incubated for 2 hrs. with various additions as indicated in the table. Preincubation with EGTA did lead to an increase in the ratio B_{2000}/B_0 which was more pronounced for ACTH than for DBCAMP. However, this ratio for 10 or 20 mU/ml ACTH was still much lower than that for 1 mU/ml ACTH. Whether membrane bound Ca^{++}, which may not have been depleted in our experiments is responsible for giving low B_{2000}/B_0 ratios with supramaximal levels of ACTH cannot be decided yet. At present we can only conclude that in the action of ACTH, Ca^{++} dependence is greater for the events before the formation of the "second messenger", cyclic AMP, than for the events following its formation.

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