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EFFECTS OF IONOPHORE A23187 ON CALCIUM FLUXES FROM CULTURED ADRENAL CELLS

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The effect of the calcium ionophore A23128 on calcium fluxes from Y-1 adrenal cortical cells was investigated. Conditions were chosen which are known to result in an inhibition of steroidogenesis ($6 \cdot 10^{-6}$ M ionophore and $3 \cdot 10^{-4}$ M extracellular calcium). Calcium efflux from Y-1 cells exhibited two distinct phases. A fast phase which was insensitive to the mitochondrial poison sodium azide and a slow, azide-sensitive phase. The ionophore brought about a rapid increase in the rate of calcium efflux and an 84% reduction in the size of the calcium pool which was associated with the slow efflux phase as well as a reduction in its rate constant. A decrease in the size of the rapidly exchanging calcium pool was also detected. Ethanol, the solvent which was used for the ionophore, slightly increased the rate constant of the rapidly exchanging pool. Conditions which resulted in diminished steroidogenic capacity also brought about a reduction in the size of an energy dependent, intracellular pool. The data is interpreted as being consistent with a hypothesis that the ionophore-induced inhibition of steroidogenesis may be causatively related to the loss of intracellular calcium or to the mechanism which brings about the loss.

Introduction

Cultured functional mouse adrenal cortical cells (Y-1) respond to a variety of adrenal secretagogues with an increase in the rate of synthesis of 20α-dihydroprogesterone [1,2]. A segment of this synthetic pathway, those reactions between the generation of cyclic AMP and the synthesis of pregnenolone, appears to be inhibited by the calcium ionophore A23187 [3,4]. This sequence of reactions has previously been demonstrated to be calcium dependent [3,5]. In view of the ability of the ionophore A23187 to alter intracellular calcium fluxes and the obligatory role of this cation in steroidogenesis, the effect of the ionophore on steriod synthesis may be a reflection of its effects on intracellular calcium distribution.

Studies performed on a variety of tissues [6–9] have revealed that the effects of the ionophore A23187 on cellular calcium distribution are very complex and are dependent upon cell type, ionophore concentration, and the calcium concentration of the extracellular fluid. The present series of experiments was conducted to determine the effects of the ionophore on Y-1 cell calcium distribution under conditions which have been previously shown to inhibit steroidogenesis.

Materials and Methods

Functional mouse adrenal cortical tumor cells (Y-1) were obtained from the American Type Culture Association. The cells were maintained in Ham's F-10 medium [10] each 500 ml of which was supplemented with: 75 ml horse serum, 12 ml fetal calf serum, 5 ml of 200 mM glutamine and 5 ml penicillin-streptomycin (5 000 I.U./ml and 5 000 μg/ml, respectively). Experiments were conducted in 60-mm tissue culture dishes (Falcon). The cells were always kept at 37°C in an incubator gassed with 95% air and 5% CO_2. $^{45}CaCl_2$ (spec. act. 20 Ci/μ calcium) was obtained from New England Nuclear, Boston, MA.

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Calcium efflux experiments under steady-state conditions. In preparation for each efflux experiment confluent plates of cells were washed twice with 3 ml of unsupplemented Ham's F-10 medium. The cells (in 3 ml unsupplemented medium) were preincubated with the indicated treatment (ionophore in 10 μl of ethanol, ethanol alone or sodium azide). After 90 min of preincubation 20 μCi of ^{45}Ca was added to each dish. With the exception of radioactively labeled calcium the content of medium was uniform throughout each experiment. After 60 min had elapsed the radioactive medium in each dish of cells was decanted and the dish was quickly washed twice with non-radioactive medium. The zero-time point for the efflux procedure corresponded to the end of the second wash. At each time point 1 ml of medium was pipetted from each dish into a scintillation vial. The medium remaining in the dish was discarded, and 1.5 ml of fresh non-radioactive medium was added. The radioactivity in each sample and that remaining within the cells at the end of the experiment was quantified with a Nulcear Chicago Uniflux III scintallation counter with ACS (Aqueous Counting Scintallant, Amersham/Searle). Efflux procedures were carried out for 180 min. At the conclusion of each experiment the number of cells on each dish was determined with a Coulter Counter (Model ZBI).

Calculations were carried out by a procedure described by Borle [11]. Starting with the ^{45}Ca content of the cells at the end of the experiment and adding the number of cpm found in each sample of medium (starting with the 180-min sample) the number of cpm remaining in the cells at each time point was determined. Data from each time point were expressed as the change in cpm per minute in the cell and were plotted against time (min). Using the graphic procedure two distinct phases of efflux were observed. The slopes and intercepts of the lines describing these two phases were calculated by least-squares linear regression analysis. Correlation coefficients revealed a close fit of the calculated line with the data points. The fast efflux phase demonstrated an r of 0.97 ± 0.01 (n = 35) while the slow phase had an r of 0.91 ± 0.01 (n = 35).

When the lines for the fast and slow phases of calcium efflux of each cell population were compared their slopes were found to differ by at least one order of magnitude. This difference in slope allowed the use of the relatively simple calculations required by a kinetic model in which the pools were considered to be in parallel. Borle [11] has demonstrated that this method of calculation would not introduce significant error even if the calcium pools were actually arranged in series. Using these procedures the slope of the line described each phase of efflux represented the rate constant of the phase while the Y-intercept divided by the specific activity of the extracellular fluid provided the calcium flux rate. The size of the calcium pool serving as the source of each efflux phase was then approximated by dividing the calcium flux rate for each phase by its rate constant.

Experiments in which steady state was perturbed by ionophore. Unsupplemented Ham's F-10 medium was added to dishes of Y-1 cells, and radioactive calcium (20 μCi ^{45}Ca) was added to the medium. After 60 min a calcium efflux procedure was carried out. Ionophore or solvent was initially added to the medium at the 60-min point of the efflux procedure. Experiments were always performed in duplicated which compared the effect of the ionophore A23187 in ethanol to the effect of ethanol alone. Efflux rate coefficient was calculated by the method of Borle [11]. The efflux rate coefficient is a measure of the amount of intracellular calcium leaving the cell at each time point. The efflux rate coefficient of the ionophore-treated cells was divided by the efflux rate coefficient from control cells at each time point and expressed as percentage of control.

Results

Perturbation of steady state

At a concentration of $6 \cdot 10^{-6}$ M the calcium ionophore A23187 caused a marked change in calcium efflux from Y-1 cells (Fig. 1). Addition of the ionophore brought about an increase in the efflux rate coefficient (a reflection of the ratio of efflux to the amount remaining in the cell) when compared to ethanol controls. In two of the three experiments the effect was immediate while in the third experiment the onset was delayed for 20 min. Once initiated the effect appeared to be brief. However, the actual duration of the effect is probably not accurately reflected by the figure. An analysis of the rates of calcium efflux from these cells revealed that the downward phase of each of the initial peaks was probably due to

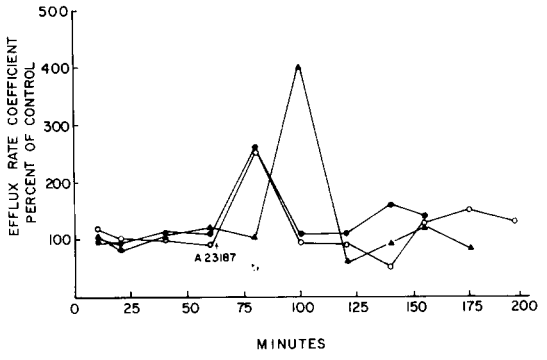

Fig. 1. Perturbation of calcium efflux by A23187. Adrenal cells were incubated in the presence of ^{45}Ca for 60 min. A ^{45}Ca efflux procedure was then performed in the manner described in Materials and Methods. The efflux rate coefficient was calculated for cell populations treated with ionophore A23187 and was expressed as percentage of the coefficient calculated from ethanol-treated cells. Ethanol and ionophore A23187 were added after 60 min of efflux and remained in the medium for the remainder of the procedures.

a depletion of intracellular ^{45}Ca. Furthermore, the later time points also appeared to be associated with small increases in the efflux rate coefficient. By adding the ionophore after 60 min of calcium exchange the effect of the compound was limited to a calcium pool which normally demonstrated slow exchangability. While this evidence suggests that an intracellular calcium pool might be altered by the ionophore A23187 the actual identity of the pool and the nature of the interaction cannot be determined by this type of experiment. For example, the apparent increase in ^{45}Ca efflux might result from a loss of intracellular calcium or from an increase in calcium influx causing an exchange between non-radioactive extracellular calcium and intracellular ^{45}Ca. Additional insight into these questions was obtained from experiments carried out under steady-state conditions.

Calcium efflux from naive and sodium azide-treated cells

Calcium efflux curves (Δcpm/Δ min versus time) from Y-1 cells displayed two distinct phases. The slowest of the two phases, representing a slowly exchanging calcium pool, exhibited a rate constant of 0.022 ± 0.001 (mean ± S.E. of 11 experiments), and the rapid efflux phase, representing a rapidly exchanging pool, had a rate constant of 0.72 ± 0.07. Technical problems precluded the quantification of a very rapid ($K > 0.75$) phase of calcium efflux. The 30-fold difference between the rate constants of the two detectable phases allowed the use of relatively simple calculations for approximating the sizes of the calcium pools from which each of the phases originated. The slow phase was found to have originated from a pool containing approx. 3.0 ± 0.3 nmol of exchangeable calcium per 10^6 cells, and the pool associated with the fast phase contained approx. 12.7 ± 2.1 nmol per 10^6 cells.

TABLE I

EFFECTS OF SODIUM AZIDE ON CALCIUM EFFLUX FROM Y-1 CELLS

Data represent rate constants and pool sizes (mean ± S.E. of three experiments) of the calcium pools associated with the fast and slow phases of the calcium efflux. The experimental cell population were treated with $1 \cdot 10^{-3}$ M sodium azide. Numbers in parentheses represent the statistical comparisons between control and azide-treated cells. n.s. indicates that sodium azide did not produce a significant effect. Statistical analysis was performed by Student's paired *t*-test.

Treatment	Phase of calcium efflux	
	Slow	Rapid
Control		
Rate constant	0.017 ± 0.0003	0.74 ± 0.13
Pool size (nmol/10^6 cells)	4.3 ± 0.65	9.9 ± 4.9
Sodium azide		
Rate constant	0.023 ± 0.001 ($p < 0.01$)	0.63 ± 0.11 (n.s.)
Pool size (nmol/10^6 cells)	2.6 ± 0.18 ($p = 0.05$)	13.5 ± 2.1 (n.s.)

Some insight into the nature of the two calcium pools was obtained by exposing the cells to a metabolic poison. Preincubating Y-1 cells for 1 h with the mitochondrial poison sodium azide ($1 \cdot 10^{-3}$ M) resulted in a reduction in the size of the slowly exchanging calcium pool as well as an increase in its rate constant. Only the slow phase had a demonstrable dependence upon mitochondrial function. Sodium azide did not produce a significant alteration in the rapid efflux phase (Table I).

Effect of the ionophore A23187 and ethanol on calcium distribution

Addition of the ionophore A23187 ($6 \cdot 10^{-6}$ M a concentration which inhibits steroidogenesis) to Y-1 cells brought about significant changes in intracellular calcium distribution. Cells which were preincubated with the ionophore accumulated only 34 ± 11% ($(1.1 \pm 0.12) \cdot 10^5$ cpm/10^6 cells) of the total amount of ^{45}Ca found in ethanol-treated cell populations. When the data were further analyzed, ionophore-induced alterations were found in the parameters which describe the calcium pools associated with both phases of efflux. When compared to ethanol controls ionophore A23187 brought about an 82% reduction in the size of the pool which was associated with the slow phase of calcium efflux and a 35% reduction in the corresponding rate constant (Table II). The ionophore also caused a significant reduction in the size of the calcium pool associated with the rapid efflux phase. At no time during the efflux procedures did the ionophore- or ethanol-treated cells lose their attachment to the plastic culture dishes.

The protocol which was followed in these experiments allowed a determination of the effect of ethanol on calcium efflux. The only significant change was a slight increase in the rate constant of the slow efflux phase when compared to naive controls (Table II).

Discussion

Exposure of Y-1 cells to the ionophore A23187 brought about marked changes in intracellular calcium distribution. This was first demonstrated in a preliminary series of experiments in which ongoing ^{45}Ca efflux was perturbed by the addition of the ionophore. The result of this maneuver was a rapid increase in calcium efflux. However, this experimental design gave no insight into the mechanism

TABLE II

EFFECTS OF THE IONOPHORE A23187 ON CALCIUM EFFLUX FROM Y-1 CELLS

Data are presented as mean ± S.E. of five experiments in which calcium efflux was measured in cells treated with the ionophore A23187 ($6 \cdot 10^{-6}$ M) dissolved in ethanol ($3 \cdot 10^{-3}$% v/v) or treated with ethanol alone. Numbers in parentheses represent the level of significance (determined by Student's paired *t*-test) when treated and ethanol controls were compared. Naive control data is shown for comparison with ethanol treated cells. Comparison of ethanol and naive cell populations (11 experiments) was performed by Student's unpaired *t*-test.

Treatment	Phase of calcium efflux	
	Slow	Rapid
Control (ethanol)		
Rate constant	0.029 ± 0.002	0.84 ± 0.16
Pool size (nmol/10^6 cells)	3.1 ± 0.6	34 ± 15
A23187 (in ethanol)		
Rate constant	0.019 ± 0.001 ($P < 0.01$)	0.56 ± 0.04 (n.s.)
Pool size (nmol/10^6 cells)	0.55 ± 0.1 ($P < 0.01$)	3.8 ± 0.6 (P=0.05)
Control (naive)		
Rate constant	0.022 ± 0.001 ($P < 0.01$)	0.72 ± 0.07 (n.s.)
Pool size (nmol/10^6 cells)	3.0 ± 0.3	12.7 ± 2.1 (n.s.)

underlying the effect other than to suggest that it reflected a change in a slowly exchanging calcium pool.

In subsequent experiments all pharmacologic manipulations were performed 1 h prior to the addition of ^{45}Ca. This experimental design allowed drug-induced changes in the calcium pools to be completed before being labelled with radioactive calcium. Alterations in calcium pool sizes were thus made evident. When this procedure was performed on naive cells two phases of calcium efflux were observed. The small rate constant (long half-life) associated with the slow efflux phase suggested that this calcium originated from an intracellular site. This interpretation was also supported by the sensitivity of this phase to sodium azide, a substance which inhibits mitochondrial but not microsomal function [12,13]. The mitochondrial poison reduced the size and increased the rate constant of the slowly exchanging calcium pool, but it had no effect on the rapid phase of efflux. Both the azide-insensitivity of the fast efflux phase and its rapid half-life suggested that this phase was superficial in origin.

Similar efflux experiments performed with the ionophore A23187 revealed reduction in the pool sizes associated with both phases of efflux. These results suggest that the rapidly developing efflux observed in the preliminary experiments was a manifestation of ^{45}Ca loss from the slowly exchanging pool, not just an increase in rate constant. The effects of the ionophore on intracellular calcium distribution suggests that this might be the basis of the inhibition of the steroidogenic pathway. Previous studies have shown that the calcium ionophore inhibits cyclic AMP-stimulated steroidogenesis but not that which is maintained by exogenous pregnenolone. These two observations indicate an inhibition of those mitochondrial reactions which are responsible for the formation of pregnenolone from cholesterol [3]. These reactions have been shown to be calcium dependent [5].

Disruption of intracellular calcium distribution is only one means by which the ionophore may inhibit steroidogenesis. At the concentration used in this study the ionophore A23187 also inhibits protein synthesis [14,15]. Stimulation of the steroidogenic pathway is known to rely on the synthesis of a short-lived protein which may mediate the transport of cholesterol to the mitochondria [16,17]. The present study demonstrated that the ionophore A23187 brings about a loss of calcium from what is in part a mitochondrial pool under conditions which are also inhibitory to steroid synthesis. The data suggest that the alterations in the azide-sensitive pool or the mechanism underlying this effect, such as its well documented ability to uncouple mitochondria [6,18,19], may contribute to the inhibitory effect of the ionophore on steroidogenesis.

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