DIVALENT CATION IONOPHORE A23187: A POTENT PROTEIN SYNTHESIS INHIBITOR

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Received September 22,1976

SUMMARY: Divalent cation ionophore A23187 has a potent inhibitory effect on protein synthesis in the C6 rat glioma cell line. Treatment with 4 μM A23187 resulted in 93% inhibition of [1-^{14}C]leucine label incorporation into proteins and a 61% increase in free pool labeling. Total RNA synthesis was not affected. Extracellular ionic calcium or magnesium are not required for these changes to occur. Therefore, these effects of A23187 may be a direct effect on protein synthesis or may result from release of internal stores of divalent cations. By comparison, ionophore X537A (4 μM) has only a slight inhibitory effect on protein synthesis.

INTRODUCTION

A23187 is a monocarboxylic acid antibiotic of low molecular weight that acts as a freely mobile carrier to transport calcium or magnesium, but not monovalent cations, from an aqueous medium buffered at pH 7.4 into a bulk organic phase (1). Two molecules of the ionophore bind a single cation to form an electroneutral lipid soluble complex that can equilibrate cation concentrations across plasma and/or mitochondrial membranes (2). X537A (Lasalocid) is also a low molecular weight monocarboxylic acid antibiotic, which in addition to transporting divalent cations has equal affinity for the alkali metal cations and H^+ as well (3). Both compounds have been widely utilized to study the influence of calcium on various cellular processes (4). We now present evidence for a potent protein synthesis

inhibition by A23187 and not X537A. These findings preclude the usage of A23187 as a tool to study the role of Ca^{++} or Mg^{++} in cellular processes involving protein synthesis, e.g., enzyme induction and cell differentiation.

We have been investigating the role of calcium in the adenosine 3',5'-monophosphate mediated induction of lactate dehydrogenase (EC 1.1.1.27) by norepinephrine in the 2B subclone of C6 cells (5), a rat glioma cell line which has retained many properties of normal nervous tissue. Maximal levels of lactate dehydrogenase are attained 24 hr after 3 μM norepinephrine treatment. We found that 24 hr exposure to 4 μM A23187 completely blocked the norepinephrine induction of lactate dehydrogenase. To ascertain whether the A23187 effect was specific for the lactate dehydrogenase induction, we also determined its effect on the non-c-AMP mediated hydrocortisone induction of glycerol phosphate dehydrogenase (EC 1.1.1.8) in the same cell line (6,7). An 89% reduction of induced levels of glycerol phosphate dehydrogenase was found.

MATERIALS AND METHODS

Cell Culture: In all the experiments described in this paper confluent monolayer cultures of C6 cells (2B subclone) were used of passage #12-18. Cells grown in Ham's F-10 medium (Gibco, Grand Island, NY) containing 10% fetal calf serum (Reheis, Chicago, IL) without antibiotics were used at 9-11 days of culture. Serum-free medium was employed during experiments.

Chemicals: L-norepinephrine bitartrate was purchased from Sigma Chemical Company. Hydrocortisone, A grade, was obtained from Calbiochem. 1[1-^{14}C] leucine and [2-^{14}C]uridine were purchased from Amersham/Searle. A23187 was the gift of Eli Lilly Company and X537A was the gift of Hoffman La Roche. Stock solutions of ionophores were made up in 5% acetone and 95% ethanol (100%)

Assays: Cyclic AMP was assayed at 20 min with a competitive protein-binding assay (8) with the charcoal modification of Brown et al. (9) and lactate dehydrogenase or glycerol phosphate dehydrogenase at 24 hours in another set of cultures using a spectrophotometric method at 340 nm wavelength (10).

[^{14}C]leucine (0.5 Ci/3 ml) incorporation into protein: 5% trichloroacetic acid (0 °C) was used to precipitate the protein. Samples were then centrifuged (0 °C) and the pellet was washed 3 times with 5% trichloroacetic acid and once with 95% ethanol (0 °C) with recentrifugation each time. The final pellet was hydrolyzed in 0.5 N NaOH for one hour at 37 °C and then counted as was the initial supernatant on a liquid scintillation counter.

Abbreviations: c-AMP adenosine 3'5'-monophosphate.

Table 1 Effects of A23187 or $LaCl_3$ on induced levels of LDH, c-AMP and GPDH in C6 cells

Treatments	Enzyme Activity (units/mg protein)	% Control	c-AMP % NE
Experiment 1 (LDH)			
Control	1661±24	100±1	
4 μM A23187	1501±51	90±3	
15 μM NE	2815±85[a]	169±5	100±4
A23187 + NE	1582±94[b]	95±6	41±2
Experiment 2 (LDH)			
Control	2333±18	100±1	
10 μM $LaCl_3$	2303±33	99±1	
3 μM NE	3804[c]	164	100±7
$LaCl_3$ + NE	3870±324	167±14	59±6
Experiment 3 (GPDH)			
Control	75±15	100±20	
4 μM A23187	98±7	131±9	
0.55 μM HC	346±12[a]	461±16	
A23187 + HC	104±4[d]	139±5	

Abbreviations: LDH, lactate dehydrogenase: GPDH, glycerol phosphate dehydrogenase; NE, norepinephrine; HC, hydrocortisone.

One unit of enzyme is defined as that amount which catalyzes the conversion of 1 nanomole of substrate/minute at 30 °C. Data for each treatment are the means ± s.d. from 3 samples. Significance: [a] $p < 0.001$ relative to control, [b] $p < 0.001$ relative to norepinephrine alone, [c] $p < 0.01$ relative to control, and [d] $p < 0.001$ relative to hydrocortisone alone.

[^{14}C]uridine (0.5 μCi/3 ml) incorporation into RNA: Harvest procedure was as in the leucine incorporation except that the hydrolyzed pellet was treated at 0 °C with 60% perchloric acid to precipitate DNA and protein; and the supernatant, containing the RNA, was read on a Gilford spectrophotometer (UV absorbance at 260 nm wavelength) and was corrected for protein contamination. An aliquot of the supernatant was counted on a liquid scintillation counter.

Protein was determined by the method of Lowry et al. (11) using bovine serum albumin as a standard.

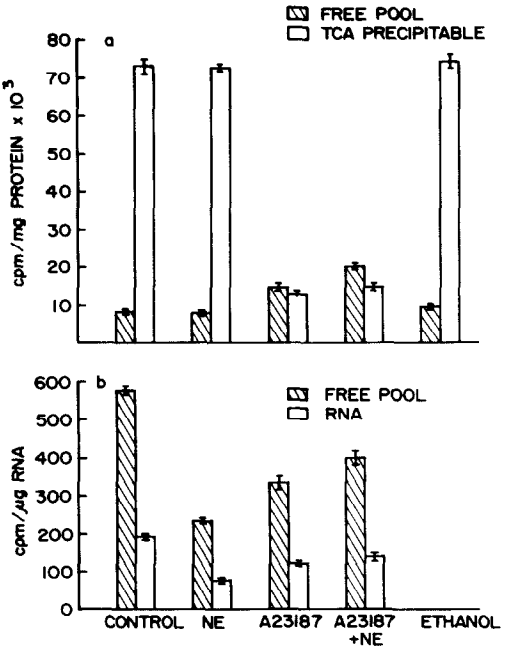

Figure 1. Effect of 4 µM A23187 and/or 3 µM norepinephrine (NE) on the incorporation of [^{14}C]leucine or [^{14}C]uridine into protein or RNA respectively. Samples were pretreated 1.5 hours (-NE) as indicated: NE was then added where indicated for 21 hours with a radioactive pulse for the last hour. a) Data for each treatment are the means ± S.D. of 3 samples. Significance: All treatments except NE or ethanol, $p < 0.001$ relative to control, and NE + A23187 relative to A23187 alone, $p < 0.001$. b) Data for each treatment are the means ± s.d. of 4 samples. Significance: All treatments, $p < 0.001$ relative to control except for (1) the RNA fraction of the NE + A23187 treatment, $p < 0.005$ relative to control or NE and (2) the free pool fraction of the A23187 treatment, $p < 0.005$ relative to NE.

RESULTS AND DISCUSSION

Table 1, experiment 1 shows that 24 hr treatment with 4 µM A23187 has no effect on basal levels of lactate dehydrogenase but completely blocks the norepinephrine induction of this enzyme. This effect was not due to a significant suppression of norepinephrine induced c-AMP levels, since 0.5 mM ethylene-glycol-bis-(aminoethyl)tetraacetate could reverse the ionophore inhibition (52%) of c-AMP levels without reversing the inhibition of lactate dehydrogenase induction (data not shown). Additionally, 10 µM lanthanum chloride inhibited norepinephrine induced c-AMP levels with no change in the

induced levels of lactate dehydrogenase (Table 1, experiment 2). Treatment with A23187 and hydrocortisone results in an 89% reduction of induced levels of this enzyme (Table 1, experiment 3).

Since both inductions were inhibited, suggesting a non-specific suppression of protein synthesis, we then examined the influence of A23187 on the incorporation of [^{14}C]leucine and [^{14}C]uridine into protein and RNA respectively. Initial experiments done with 20 hours of exposure to A23187 and norepinephrine, with a radioactive pulse the last hour, indicated an 82% depression of TCA precipitable counts and a 70% increase in the free pool labeling (Figure 1a). Since the free pool count was increased, the depression of TCA insoluble counts was not simply due to reduced precursor levels. A 40% drop in uridine incorporation into RNA was paralleled by a 40% drop in the free pool labeling with ionophore treatment (Figure 1b). Norepinephrine treatment also resulted in a significant decrease (60%) of free pool and RNA labeling. Thus, the ionophore was not reducing leucine transport nor total RNA formation, but rather was inhibiting protein synthesis at some subsequent point.

These initial experiments involved long exposure times to the ionophore and thus a time course experiment was done (Table 2, experiment 1) to determine effects at earlier times. Maximal inhibition (93%) was seen at the earliest time point: simultaneous ionophore treatment with the [^{14}C]leucine pulse for one hour. In a separate experiment (Table 2, experiment 2), the increase in free pool labeling and decrease in protein fraction counts is again seen at one hour exposure to A23187, while with 4 μM X537A there is a small decrease (12%) in protein counts and a small increase (11%) in a free pool labeling. Longer exposure (21 hours) to 4 μM X537A reveals no significant changes in labeling. When exposed to 20 μM X537A for one hour the cells were crenated, very granular and there was some cell loss; so that these decreases

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Abbreviations: TCA, trichloroacetic acid.

Table 2 Effect of various exposure times to ionophore on [^{14}C]leucine incorporation into protein

Incubation time (hrs)	Ionophore (μM)		Protein fraction cpm/mg protein (x10^{-3})	% Control (mean)	Free pool cpm/mg protein (x10^{-3})	% Control (mean)
Experiment 1: 1	0		35.65±2.16	100	11.24±0.65	100
1	4	A23187	2.59±0.38[a]	7	16.02±0.96[a]	143
4	0		44.90±1.67	100	11.87±0.51	100
4	4	A23187	6.83±0.95[a]	15	16.65±0.07[a]	140
14	0		89.81±7.00	100	16.69±0.75	100
14	4	A23187	25.80±1.19[a]	29	25.48±1.14[a]	153
21	0		111.09±8.62	100	15.58±1.18	100
21	4	A23187	30.96±2.05[a]	28	27.12±1.26[a]	174
Experiment 2: 1	0		48.23±5.31	100	7.24±0.33	100
1	4	A23187	3.99±0.53[a]	8	10.97±0.41[a]	152
1	4	X537A	37.60±4.44[b]	78	8.04±0.43[b]	111
1	20	X537A	3.36±0.28[a]	7	1.61±0.45[a]	22
21	0		147.98±6.77	100	26.91±12.13	100
21	4	X537A	141.95±8.82	96	26.35±5.83	98

[^{14}C]leucine (0.5 μCi/3 ml) was added for the last hour of incubation (37 °C). Data for each treatment are the means ± s.d. of 4 samples. Significance: [a]$p < 0.001$ relative to control; [b]$p < 0.025$ relative to control.

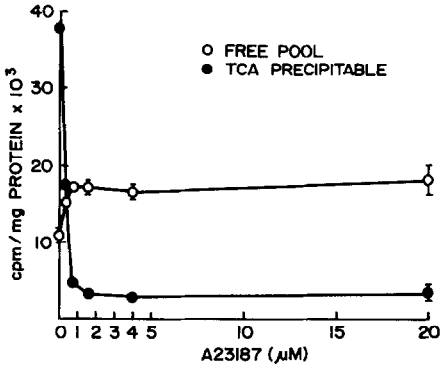

Figure 2. Dose-dependence of A23187 effect on [^{14}C]leucine incorporation into protein. A23187 at the indicated concentrations was added simultaneously with [^{14}C]leucine (0.5 μCi/3 ml) for a one hour incubation (37 °C). Data for each treatment are the means ± s.d. of 4 samples. Where the standard deviation is not shown, it is smaller than the size of the symbol. Significance: All treatments $p < 0.001$ relative to control except 20 μM A23187 (free pool) with $p < 0.005$ relative to control.

in counts are probably toxic effects, particularly since 20 hour exposure to 20 μM X537A resulted in complete cell death.

A dose response determination (Figure 2) indicated maximal inhibition (92%) of [^{14}C]leucine incorporation into protein with 4 μM A23187, with substantial inhibition (88%) at only 0.8 μM A23187. Figure 2 also shows the maximal increase (61%) of free pool labeling at 0.8 μM A23187, plateauing thereafter.

Using 4 μM A23187 simultaneously with a one hour [^{14}C]leucine pulse in calcium or magnesium free medium results in label distribution identical to that of complete medium (data not shown), suggesting no requirement for extracellular ionic calcium or magnesium. Release of mitochondrial stores of divalent cations cannot be ruled out, but is weakened by the minimal effect of X537A, which can also equilibrate these ions across membranes.

In conclusion, A23187 is now shown for the first time to be a potent protein synthesis inhibitor at very low concentrations. This may be a direct effect on protein synthesis or may result from release of internal stores of divalent cations. In addition, the ionophore apparently increases leucine

and decreases uridine transport into the cells. Therefore, in some experimental conditions using intact cells, the effects of A23187 may not be solely attributable to increased permeability of plasma membranes to divalent cations as previously claimed.

ACKNOWLEDGEMENTS

We thank Diane Inglish for expert technical assistance. This work was supported by ERDA Contract E(04-1)GEN-12 and USPHS Grant HD-05615. J. E. Bottenstein is an NIMH-MHTP predoctoral trainee (5T01MH06415).

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