INHIBITION OF CELLULAR GROWTH AND NUTRIENT TRANSPORT INDUCED BY CALCIUM IONOPHORE A23187 IN MASTOCYTOMA P-815 CELLS

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Accepted February 28, 1983

Abstract—Kinetic analysis of Ca\(^{2+}\) metabolism in mastocytoma P-815 cells showed that at non-cytotoxic and growth-inhibitory concentrations (<10 \(\mu\)g/ml), the calcium ionophore A23187 did not affect the rate constant (36.2 min\(^{-1}\)) or the compartment size (8.32 nmol/10\(^6\) cells) of the fast phase of calcium exchange at the cell surface. In contrast, A23187 (1 to 10 \(\mu\)g/ml) dose-dependently increased the compartment size of slow phase I of the intracellular calcium uptake from 0.54 to 2.39 nmol/10\(^6\) cells without affecting the rate constant (2.38 min\(^{-1}\)). In addition, A23187 (5 to 10 \(\mu\)g/ml) produced an additional compartment [slow phase II] with a rate constant of 0.25 min\(^{-1}\) and increased its compartment size from 0.42 to 1.26 nmol/10\(^6\) cells. A23187 (10 \(\mu\)g/ml) in the presence of Ca\(^{2+}\) (0.9 mM) non-competitively inhibited the membrane transport of amino acids, glucose and nucleosides; and it also reduced the cellular ATP level and the membrane fluidity. Apparently, A23187 inhibits the growth of mastocytoma cells by elevating the intracellular concentration of Ca\(^{2+}\), which in turn reduces the membrane fluidity and the carrier-dependent as well as the active membrane transport of various nutrients required for cellular growth.

It is generally believed that a variety of cellular functions including cellular growth are mediated by the increments in the intracellular concentration of Ca\(^{2+}\); and this hypothesis is strongly supported by the experiments with divalent cation ionophores such as A23187 (1) and Ca\(^{2+}\), which showed the elevation of DNA synthesis in lymphocytes (2, 3) and parthenogenesis of sea urchin eggs (4). In contrast, A23187 in the presence of Ca\(^{2+}\) inhibits the growth of mastocytoma P-815 cells (5). Based on kinetic analysis using Borle's three compartment closed system (6), we have observed the presence of two kinetically distinct compartments of Ca\(^{2+}\), fast and slowly exchangeable, in exponentially growing mastocytoma P-815 cells (7). In order to examine further the effect of intracellular Ca\(^{2+}\) uptake on cell growth, in the present study, we have investigated the kinetics of Ca\(^{2+}\) transport induced by A23187 in cultured mastocytoma P-815 cells and also examined the Ca\(^{2+}\)-dependency of the transport systems of several nutrients (amino acids, glucose and nucleosides) required for cell growth.

Materials and Methods

Cells and cell culture: Mouse mastocytoma P-815 cells were maintained in a suspension culture at 37\(^\circ\)C in Fischer-Sartorelli's medium supplemented with 5% fetal calf serum. The cell culture and the cell number determination were performed as described previously (7). The cells in the exponential growth phase were harvested and washed three times at 37\(^\circ\)C with a medium containing 137 mM NaCl, 4.15 mM KCl, 0.9 mM CaCl\(_2\), 1.05 mM
MgCl₂ and 10 mM 4-(2-hydroxyethyl)-1-piperazinoneethanesulfonic acid (Hepes) adjusted to pH 7.4 with NaOH (Hepes medium).

**Cell viability:** The nigrosin (0.2% in Hank's solution) staining test of Kaltenbach et al. (8) was used to determine the cell viability. In addition, the discharge of cellular lactate dehydrogenase into the medium was also estimated according to Ellis et al. (9).

**DNA synthesis:** Mastocytoma P-815 cells suspended in Hepes medium (0.5x10⁶/ml) were incubated with [methyl-3H]thymidine in the presence or absence of various concentrations of A23187 (dissolved in dimethyl sulfoxide, 1 mg/ml, as a stock solution) at 37°C for 30 min. The radioactivity in the acid-insoluble fraction was counted as described previously (10).

**45Ca²⁺ influx measurements:** Cells suspended in Hepes medium (5x10⁶ cells/ml) were preincubated with or without A23187 (10 μg/ml, 19 nM) at 37°C for 10 min. The Ca²⁺ uptake assay was initiated by the addition of 45Ca²⁺ (final specific activity, 0.6 Ci 45Ca²⁺/mol Ca²⁺), followed by incubation at 37°C, unless otherwise noted. At various times (5 sec to 30 min) after 45Ca²⁺ addition, aliquots (0.1 ml) of the incubation mixture were filtered using HAWP 0.45 μm Millipore filters. The filters were rinsed three times with cold 0.25 M sucrose (2.5 ml) in deionized double distilled water, dried, dissolved in ethylene glycol monomethyl ether (2 ml) and counted in 0.5% (w/v) 2,5-diphenyloxazole in toluene (10 ml). Since it was rather difficult to estimate calcium uptake of the fast phase independently of that of the slow phase, kinetic parameters (half time, rate constant, flux and compartment size) were calculated from semilogarithmic replots of [(maximal calcium uptake of both phases, Xₘₐₓ) – (calcium uptake of both phases at any given time, Xₜ)] (expressed as nmol calcium/10⁶ cells) against time as described previously (7). The calcium uptake at 30 min at 37°C was taken as being maximal, representing the sum of uptakes of both phases at equilibrium.

**Measurements of transport of nutrients (amino acids, glucose and nucleosides):** (a) Effect of A23187: Cells (5x10⁶ cells) suspended in Hepes medium (0.5 ml) were preincubated at 37°C for 10 min in the presence or absence of A23187 (10 μg/ml). Then, the cell suspensions were incubated for 1 min at 37°C with 0.2 to 5.0 mM [¹⁴C]-histidine, [³H]-proline, [³H]-lysine, [³H]-glutamate, [¹⁴C]-D-glucose, [³H]-thymidine or [³H]-uridine (1.0 mCi/mmol) for Lineweaver-Burk replot analysis. (b) Amino acid transport systems: Cell suspensions in Hepes medium (5x10⁶ cells/0.5 ml) were incubated for 1 min at 37°C with 0.1 mM labeled histidine, proline, lysine or glutamate (1.0 mCi/mmol) in the presence of various non-labeled amino acids (10 mM, except for tyrosine, 3 mM). (c) Na⁺-dependency: Cell suspensions in Hepes medium containing 140 mM choline-HCl instead of 140 mM NaCl (5x10⁶ cells/0.5 ml) were incubated for 1 min at 37°C with various labeled nutrients (1 μCi/0.02–0.2 mM). The reactions (a, b and c) were terminated by chilling on ice. After washing twice with cold Hepes medium, cell pellets were extracted with cold 5% perchloric acid (0.3 ml). Aliquots (0.2 ml) of the cell extracts were assayed for radioactivity in a scintillant [10 ml, 0.5% 2,5-diphenyloxazole in toluene/Triton X-100 (2:1, v/v)].

**Determination of ATP:** Cellular ATP was determined by the luciferin-luciferase method of Bihler and Jeanrenaud (11).

**Electron spin resonance spectroscopy:** Mastocytoma cells (5x10⁶ cells/0.4 ml of Hepes medium) were incubated with 5-doxylstearic acid (6 μl, 1.7 mg dissolved in 1 ml of 25% ethanol) for 45 min at room temperature, washed three times with Hepes medium, and then resuspended in the same
medium. Aliquots (50 µl) of the spin-labeled cells were withdrawn and put into siliconized capillaries which were then sealed. Electron spin resonance (ESR) spectra were obtained with a Japan Electron Optics Laboratory model JES-FE3X (Tokyo, Japan) (scan range, 50 G; field setting, 3270 G; time constant, 0.1 sec; scan time, 4 min; modulation amplitude, 1 G; modulation frequency, 106 KHz; microwave power 20 milliwatts; and microwave frequency, 9.199 GHz). All spectra were recorded at 37°C. The following order parameter (S) was used to evaluate the membrane flexibility:

$$S = \frac{T_{zz} - T_{xx}}{2T_{11} + 2T_{12}}$$

$2T_{zz}$ and $2T_{xx}$ are the outer and inner hyperfine splittings. $T_{xx}$ and $T_{zz}$ are the hyperfine splitting elements of the static interaction tensor (I) parallel to the static Hamiltonian (H) principal nuclear hyperfine axes x and z, respectively (12).

Materials: The following materials were purchased from the sources indicated: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) and ethyleneglycol bis(β-aminoethyl ether)-N,N′-tetraacetic acid (EGTA), Nakarai Pure Chemicals (Kyoto, Japan); ionophore A23187, Calbiochem-Boehring Corp. (La Jolla, CA, U.S.A.); [methyl-3H]thymidine (43 Ci/mmol), [5,6-3H]uridine (46 Ci/mmol), L-[14C(U)]histidine (300 mCi/mmol) and D-[14C(U)]glucose (230 mCi/mmol), Amersham International (Amersham, Buckinghamshire, England); 45CaCl2 (0.35 Ci/mol), [3H(G)]proline (5 Ci/mmol), [2,3-3H]glutamic acid (23.3 Ci/mmol) and [4,5-3H(N)]lysine (80.5 Ci/mmol), New England Nuclear (Boston, MA, U.S.A.); and 5-doxylstearate, Syva Co. (Palo Alto, CA, U.S.A.). Other chemicals of reagent grade were obtained commercially.

Results

Effect of A23187 on DNA synthesis and growth of mastocytoma P-815 cells: In the presence of 0.9 mM Ca2+, A23187 ($M_r=532$) dose-dependently inhibited DNA synthesis (Fig. 1A) and cellular division (Fig. 1B). EGTA (1 mM) completely abolished the inhibitory effect of the ionophore on DNA synthesis. The ID₅₀'s of A23187 for inhibition of DNA synthesis (37°C, 30 min) and cellular growth (37°C, 12 hr) were 3.4 and 4.3 µM (1.8 and 2.2 µg/ml), respectively.

In the cells treated with A23187 (up to
10 μg/ml, 19 μM) at 37°C for 12 hr, the percentage of non-viable cells was less than 7%, as compared with about 2% in the untreated cells. Furthermore, this growth-suppressing effect of A23187 was reversed by washing the treated cells (19 μM, 37°C, 12 hr) and resuspending them in fresh medium without the ionophore. The washed cells began to multiply almost as normally as untreated cells. Although A23187 at concentrations lower than 19 μM was not significantly cytotoxic for mastocytoma P-815 cells, at 20 μM and 30 μM, it not only completely suppressed the cell growth (37°C, 12 hr), but also caused significant increases of stained cells (25% and 45%, respectively) and lactate dehydrogenase leakage (23% and 38%, respectively).

Effect of A23187 on Ca²⁺ uptake into mastocytoma P-815 cells: In the presence of A23187 (10 μg/ml), the Ca²⁺ uptake into mastocytoma P-815 cells at 37°C proceeded in two phases, an initial fast phase followed by a slow phase, and gradually reached the maximum level in about 10 min (Fig. 2 inset), in contrast to the less than 2 min observed in the absence of the ionophore (7). Semilog replots of the data showed that the Ca²⁺ uptake of the slow phase, which represented the equilibrium of medium and intracellular Ca²⁺, consisted of two distinct components, slow phases I and II (Fig. 2), instead of a single component in the absence of the ionophore (7). Kinetic analyses of Ca²⁺ uptake were then performed with cells pretreated with various concentrations of A23187 for 10 min at 37°C. As shown in Table 1, the rate constant and compartment size of Ca²⁺ uptake of the fast phase, which represented Ca²⁺-binding to the cell surface, were not affected by increasing concentrations of A23187. On the other hand, the Ca²⁺ uptake of the slow phase was separated into two components in the presence of 5 μg/ml or higher concentrations of the ionophore.

Slow phase I had about the same rate constant as that of the slow phase of A23187-untreated cells, while slow phase II showed about 1/10 of the value of the control cells. On the other hand, the compartment size of these two slow phases increased progressively with increasing concentrations of A23187. Based on these results and the cell viability data mentioned above, the ionophore was used at 10 μg/ml (19 μM) in most of the following experiments.
At 0°C, where the cell membrane is assumed to take on a crystalline conformation, thus restricting the free movement of A23187, values for the kinetic parameters of Ca\(^{2+}\) uptake of the fast phase were not significantly different from those obtained at 37°C, while values of slow phase I became lower than those of the 37°C control (rate constant, 2.38 to 2.06 min\(^{-1}\); compartment size, 0.54 to 0.23 nmol/10^6 cells). Furthermore, the additional Ca\(^{2+}\) compartment, slow phase II produced by A23187 at 37°C, disappeared at 0°C.

**Table 1. Effect of A23187 on parameters of Ca\(^{2+}\) uptake into mastocytoma P-815 cells**

| A23187 (μg/ml) | 0 (control) | 1 | 2.5 | 5 | 7 | 10 |
|---------------|-------------|---|-----|---|---|----|
| Half time (min) | 0.019 | 0.019 | 0.019 | 0.020 | 0.020 | 0.019 |
| Rate constant (min\(^{-1}\)) | 36.2 | 36.9 | 36.5 | 34.8 | 33.9 | 36.3 |
| Flux (nmol/10^6 cells/min) | 301 | 302 | 300 | 283 | 272 | 286 |
| Compartment size (nmol/10^6 cells) | 8.32 | 8.41 | 8.21 | 8.12 | 8.01 | 7.89 |

Various kinetic parameters of the calcium compartments in the presence of 0 to 20 μg/ml of A23187 were obtained from semilog plots of the data as described under Materials and Methods. Each value represents the mean of two determinations with triplicate samples at each point.

Effect of A23187 on nutrient transport:

(a) Amino acid transport: Of the four representative amino acids tested, the transport of proline, a neutral amino acid, was inhibited almost completely (by 93–100%) by glycine, alanine, serine, cysteine, glutamine, asparagine, methionine and histidine, but not by glutamate, aspartate, lysine or arginine. The transport of glutamine, an acidic amino acid, was moderately (by 10–40%) inhibited by aspartate and several other amino acids, but not by lysine, arginine or histidine (data not shown). These results indicate that several transport systems (A-, Ly\(^{+}\)- and L-systems) for amino acids are present in mastocytoma P-815 cells as reported for other mammalian cells (13, 14). Table 2 summarizes kinetic constants for transport of these four amino acids and inhibitor constants of typical competitive inhibitors calculated from Lineweaver-Burk equations.

Based on these results, the effect of A23187 and Ca\(^{2+}\) on the transports of these amino acids were investigated by Lineweaver-Burk plot analysis (Fig. 3A–3D, Table 2). In the absence of A23187, the extracellular Ca\(^{2+}\) had no effect on K\(_{m}\) and V\(_{max}\) values of these transports. A23187 strongly and non-competitively inhibited these transports in the presence of Ca\(^{2+}\), decreasing the V\(_{max}\). A23187 was more inhibitory at 2.0 mM Ca\(^{2+}\) than at 0.9 mM Ca\(^{2+}\). EGTA (1.0 mM)
completely abolished these inhibitory actions of the ionophore. Replacement of NaCl with choline-Cl in Hepes medium resulted in a significant suppression of the transport of histidine and proline. On the other hand, this replacement suppressed the transport of lysine and glutamate by only 21% and 25%, respectively (Table 3). (b) Glucose transport: The inhibition of glucose transport by A23187 was also Ca²⁺-dependent and non-competitive as in the case of amino acid transport (Table 2). By replacement of NaCl with choline-Cl, the glucose uptake was suppressed by 36% (Table 3). (c) Thymidine and uridine transport: A23187 in the presence of Ca²⁺ also non-competitively inhibited the uptake of both thymidine and uridine. As in the case of transport of amino acids and glucose, EGTA (1.0 mM) completely abolished the inhibitory effect of A23187. By replacing NaCl with choline-Cl, the uptake of thymidine and uridine was suppressed by 12% and 36%, respectively (Table 3).
presence of 0.9 mM Ca$^{2+}$ in 5 min at 37°C. EGTA (1.0 mM) completely abolished this inhibitory effect of A23187 (Fig. 4).

**Effect of A23187 on membrane fluidity:**
A23187 Ca$^{2+}$ concentration-dependently increased the order parameter (S) of ESR.

Table 2. Effect of A23187 and Ca$^{2+}$ on transport of amino acids, glucose and nucleosides in mastocytoma cells

| Amino acid | Control | EGTA (1.0 mM) | CaCl$_2$ (0.9 mM) | CaCl$_2$ (2.0 mM) |
|------------|---------|---------------|-------------------|-------------------|
| 1) Histidine | $K_m$ | 0.303 | 0.304 | 0.304 | 0.309 |
|            | $V_{max}$ | 5.62 | 5.60 | 4.08 | 3.33 |
| valine     | $K_i$ | 2.68 | 1.00 | 1.00 | 1.00 |
| 2) Proline  | $K_m$ | 2.38 | 2.35 | 2.41 | 2.40 |
|            | $V_{max}$ | 3.71 | 3.80 | 1.61 | 1.00 |
| alanine    | $K_i$ | 2.10 | 0.74 | 0.74 | 0.33 |
| 3) Lysine   | $K_m$ | 0.345 | 0.342 | 0.340 | 0.352 |
|            | $V_{max}$ | 2.13 | 2.14 | 0.74 | 0.33 |
| arginine   | $K_i$ | 0.394 | 0.74 | 0.74 | 0.33 |
| 4) Glutamate | $K_m$ | 0.159 | 0.161 | 0.160 | 0.152 |
|            | $V_{max}$ | 1.16 | 1.21 | 0.89 | 0.37 |
| aspartate  | $K_i$ | 2.50 | 0.89 | 0.89 | 0.37 |
| D-Glucose  | $K_m$ | 2.94 | 2.91 | 2.90 | 2.88 |
|            | $V_{max}$ | 1.35 | 1.41 | 0.14 | 0.11 |
| Nucleoside |       |       |       |       |
| 1) Thymidine | $K_m$ | 0.086 | 0.081 | 0.088 | 0.086 |
|            | $V_{max}$ | 0.087 | 0.089 | 0.039 | 0.020 |
| 2) Uridine  | $K_m$ | 0.25 | 0.25 | 0.23 | 0.26 |
|            | $V_{max}$ | 0.077 | 0.079 | 0.054 | 0.035 |

$K_m$, $K_i$: mM, $V_{max}$: nmol·min$^{-1}$·10$^6$ cells$^{-1}$

$K_m$, $V_{max}$ and $K_i$ were calculated from Lineweaver-Burk replots of the data of nutrient transport assays as described under Materials and Methods. Each value represents the mean of three determinations.

Table 3. Effect of replacement of NaCl with choline chloride on the nutrient transport in mastocytoma cells

| Medium containing | (a) | (b) |
|-------------------|-----|-----|
| NaCl 70 mM        |     |     |
| Choline-HCl 70 mM  |     |     |

|    | (a) | (b) |
|----|-----|-----|
|    | 42  | 98  |
| Histidine |     |     |
| Proline   | 21  | 59  |
| Lysine    | 4   | 21  |
| Glutamate | 12  | 25  |
| D-Glucose | 19  | 36  |
| Thymidine | 12  | 12  |
| Uridine   | 36  | 36  |

% of inhibition

Experimental conditions were similar to those for control cells in Table 2, except for the replacement of NaCl (140 mM) in the media with choline-Cl. Percent of inhibition was calculated from the $V_{max}$ value of control cells in the NaCl medium in Table 2 and that in the choline-Cl-containing medium.
signals of the cellular membrane spin-labeled with 5-doxylstearic acid (Fig. 5). On the other hand, in the presence of 1.0 mM EGTA and in the absence of Ca\(^{2+}\), the ionophore did not alter the order parameter, giving the same value as the control cells incubated in the presence of CaCl\(_2\) without A23187.

Discussion

The doses of A23187 that induce measurable increases in Ca\(^{2+}\) uptake are not always optimal for stimulating cellular functions as observed for pig or mouse lymphocytes (15). In addition, A23187 is also known to be dose-relatedly cytotoxic for rat or mouse thymocytes in the presence of Ca\(^{2+}\) (16–18). However, A23187 at up to 19 \(\mu\)M was not significantly cytotoxic for mastocytoma P-815 cultured for 12 hr at 37°C as estimated by both nigrosin-staining and lactate dehydrogenase leakage tests and also by the reversibility of its inhibitory effect on the cellular growth. Thus, the effect of A23187 on the nutrient uptake and on the ATP level of mastocytoma P-815 cells observed in the concentration range used in the present study are probably not due to the non-specific cellular damage caused by the ionophore.

The kinetic analysis of Ca\(^{2+}\)-uptake into mastocytoma P-815 cells using the three compartment closed system of Borle (6) indicated that A23187 had little effect on the Ca\(^{2+}\)-binding to the cell surface (fast phase of Ca\(^{2+}\) uptake), but it affected the intracellular calcium uptake (slow phase I of Ca\(^{2+}\) uptake) by increasing the flux and compartment size, and it maintained the rate constant to the same level as observed in the absence of the ionophore. Apparently, A23187 affects the Ca\(^{2+}\) transport system across the plasma membrane, elevating the membrane permeability to Ca\(^{2+}\) and causing an increase of the compartment size of slow phase I. The appearance of a second compartment in the slow phase (slow phase II) might be due to the Ca\(^{2+}\) carrier activity of A23187 itself. The disappearance of these A23187 effects at 0°C seems to support the above assumption, indicating the dependence of the mobility and action of the ionophore on the fluidity of the plasma membrane.

Studies of cellular nutrient uptake in mastocytoma P-815 cells indicated that as observed for various mammalian cells and membrane vesicles (19–23), amino acids, glucose and nucleosides were taken up by external Na\(^{+}\)-independent as well as Na\(^{+}\)-dependent processes. In the presence of Ca\(^{2+}\), A23187 inhibited rather non-specifically both Na\(^{+}\)-independent and -dependent transport systems for cellular nutrients. EGTA completely abolished these inhibitory effects of A23187.

Furthermore, as observed for human erythrocytes (24), A23187 Ca\(^{2+}\)-dependently reduced the cellular concentration of ATP in mastocytoma cells (Fig. 4). A23187 is also known to uncouple the oxidative phosphorylation in the hepatic mitochondria of rats (25) and to decrease the concentration...
of phosphocreatine and ATP/ADP ratio in rat muscle (26). Since the transmembranous Na\(^+\)-gradient as the driving force for the active transport is maintained by the Na\(^+\), K\(^+\)-ATPase of the plasma membrane (27, 28), A23187-induced reduction of cellular ATP levels may further augment the decrease of nutrient uptake.

A23187 also Ca\(^{2+}\)-dependently decreased the membrane fluidity of mastocytoma cells (Fig. 5). A similar Ca\(^{2+}\)-induced decrease of membrane fluidity was reported in the cortex fraction isolated from fertilized sea urchin eggs compared with that from unfertilized ones (29). Ca\(^{2+}\) might directly interact with the head groups of membrane phospholipids (30) or with cytoskeletons (31). It is also possible that the reduction of cellular ATP levels by A23187 induces the decrease of membrane fluidity. Elucidation of the exact mechanism by which the membrane fluidity is altered by the intracellular Ca\(^{2+}\) or cellular ATP level awaits further examination.

Since A23187 in the presence of extra-cellular Ca\(^{2+}\) had no effect on the affinity (K_m) of the cells for the nutrients, but rather reduced the maximal rate of their uptake (V_m) (Table 2, Fig. 3), the ionophore probably exerted its inhibitory effects on the areas adjacent to the transport carriers and not directly on the carrier molecules or nutrient substances. This inhibitory action of the ionophore was completely abolished by the removal of Ca\(^{2+}\) with EGTA. Therefore, it seems to be reasonable to assume that the ionophore binds to the membrane, induces the elevation of intracellular Ca\(^{2+}\) by increasing the membrane permeability to Ca\(^{2+}\) but not by releasing Ca\(^{2+}\) from mitochondria or endoplasmic reticulum, reduces the cellular level of ATP and alters the membrane fluidity (Fig. 5), eventually causing the inhibition of membrane transport of nutrients required for cellular growth.

Acknowledgments: We wish to thank Yuko Ueha for her assistance in preparing the manuscript. This work was supported in part by grants from the Ministry of Education, Science and Culture of Japan.

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