Rapid stopping of A23187 action by phosphatidylcholine

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The action on pigeon erythrocytes of the Ca^{2+} ionophore, A23187, can be nearly completely stopped within 30 s by treatment with a phosphatidylcholine dispersion at 39°C. A time-limited $^{45}Ca^{2+}$ uptake pulse can be produced by treating cells sequentially with A23187 and lipid, and the time-course of expulsion of this $^{45}Ca^{2+}$ uptake pulse can be easily determined.

Ca^{2+} flux *A23187 removal* *Phospholipid* *Erythrocyte*

1. INTRODUCTION

Ionophores are widely used to adjust ionic compositions within cells, subcellular organelles and membrane vesicles [1]. For many purposes it would be desirable to remove them again. The divalent cation ionophore, A23187, can be removed from erythrocytes by treatment with serum albumin and washing [2]. We have reported [3] that the action of the Na^+,K^+-ionophore, gramicidin D, on pigeon erythrocytes could be nearly stopped by incubating gramicidin-treated cells with phospholipid vesicles for 10 min at 39°C. We now report that the action of large doses of A23187 on pigeon erythrocytes is virtually completely stopped in < 30 s by the addition of phospholipid vesicles at 39°C. Temporally controlled Ca^{2+} entry pulses can be produced by sequential additions of ionophore and lipid.

2. MATERIALS AND METHODS

$^{45}CaCl_2$ and NaB^3H_4 were from ICN (Irvine CA). Tritium-labeled maltitol was prepared by reduction of maltose with NaB^3H_4 [4] and purified by passage through a Sephadex G-15 column. The scintillation counting cocktail 3a70B was from RPI (Mount Prospect IL), A23187 was a gift from Eli Lilly (Wood's Hole MA), α-tocopherol was from Sigma (St Louis MO) and egg yolk phosphatidylcholine was from Avanti (Birmingham AL).

Pigeon erythrocytes obtained as in [3] were washed 3 times with 132 mM NaCl, 10 mM TES (pH 7.4), 10 mM D-glucose and 2 mM $MgCl_2$. Cells were incubated in the same buffered saline plus 3 mM potassium phosphate and $^{45}Ca^{2+}$ at the desired $[Ca_o^{2+}]$. In some experiments amino acids were added (8.4 mM A, 6 mM N, 0.52 mM C*, 6 mM Q, 1 mM P, 5.2 mM S and 2.8 mM T). A23187, 2 mM in ethanol–dimethylsulfoxide (7:1, v/v) was used for the dose of 60 μmol/kg cells, 0.1 mM in ethylene glycol–dimethylsulfoxide (2:1, v/v) was used for doses of 4–10 μmol/kg cells. Equal volumes of solvent blanks were added to control samples. The phosphatidylcholine dispersion was prepared as follows. Lipid (20 mg/ml hexane) was mixed with α-tocopherol (1% of the lipid) and the hexane removed with a stream of N_2. Then 200 mg lipid and 1 ml buffered saline were sonicated under N_2 in a 12°C bath for five 30-s periods at setting 3 with a microtip (sonicator Model 185D, Heat System Ultrasonic, Plainview NJ); 40 mg lipid/g cells were used. Without α-tocopherol, cells turn brown after

* Cysteine was prepared by reducing cystine with the stoichiometric amount of dithiothreitol since commercial cysteine inhibited Ca^{2+} transport by cytoplasmic membrane vesicles from pigeon erythrocytes [5]

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30 min at 39°C. In one experiment, the lipid suspension was held at 39°C for 10 min, then held at 0°C until use. This lipid was virtually ineffective. Cell suspensions were incubated for various times and aliquots (0.1 g cells) were mixed with ice-cold diluent (fig.1,2) containing [^{3}H]maltitol and centrifuged promptly. The lipid does not sediment. Cell pellets were extracted with 1.0 ml 8.5% trichloroacetic acid, 5 mM $CaCl_2$ and 0.50 ml of the trichloroacetic acid extracts were mixed with 7.0 ml 3a70B counting cocktail and radioactivities measured by liquid scintillation counting. Pellet ^{45}Ca counts were corrected for ^{45}Ca from entrained medium using pellet ^{3}H counts and the ^{45}Ca:^{3}H ratio in the medium.

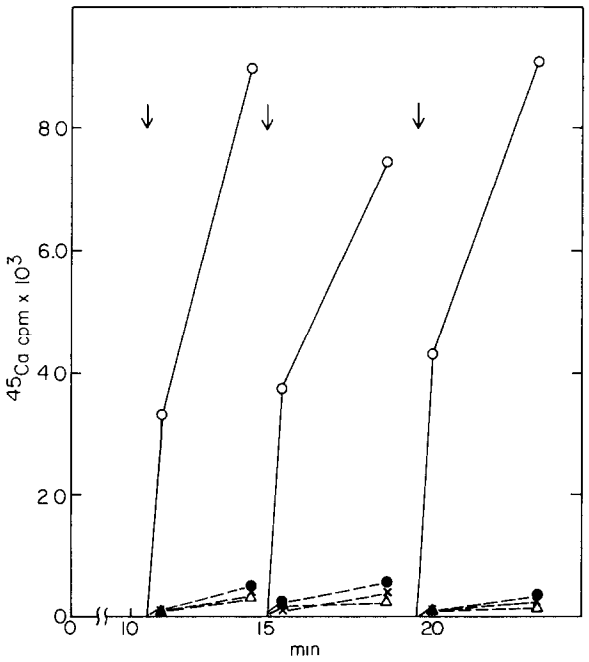

Fig.1. The effect of phosphatidylcholine addition on A23187 action. Cells (50%, w/v) in buffered saline, with 'trace' Ca^{2+} were incubated at 39°C with or without the addition of 60 μmol A23187/kg cells. After 10 min, 0.1 vol. lipid or buffered saline was added and tracer $^{45}Ca^{2+}$ (1.4 nM, 1.0 μCi/ml) was added at the times shown by the arrows. Aliquots of 0.20 ml were withdrawn 0.5 and 4.0 min after ^{45}Ca addition and mixed with 1.3 ml ice-cold buffered saline containing [^{3}H]maltitol (0.4 mM, 0.3 μCi/ml) and centrifuged immediately for 10 s in a Beckman microfuge at 8730 × *g*. A23187 added: + PC (●), − PC (○). No A23187 added: + PC (△), − PC (×).

3. RESULTS AND DISCUSSION

The action of a large dose of A23187 was stopped at 39°C < 30 s by the addition of 0.1 vol. lipid dispersion (fig.1). The suppression of A23187 action was the same with lipid added 0.5, 5 or 10 min before the $^{45}Ca^{2+}$ tracer. With the 'trace' [Ca^{2+}] (~5 μM) used in this experiment, there was a just perceptible difference between $^{45}Ca^{2+}$ uptake by cells never exposed to A23187 and cells treated with A23187 and then lipid. At [Ca_o^{2+}] = 0.50 mM $^{45}Ca^{2+}$ uptake in 1 min was 169 μmol/kg (not shown). This uptake underestimates the influx, which was too fast to measure by our procedure.

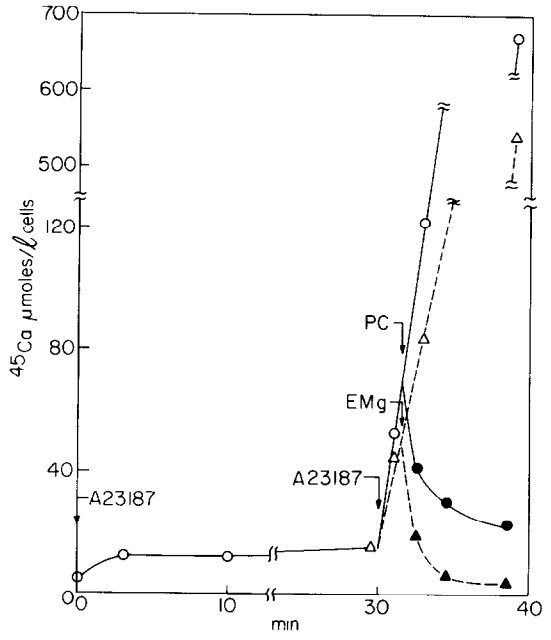

Fig.2. Loss of cell $^{45}Ca^{2+}$ following addition of phosphatidylcholine or EGTA. Cells (20%, w/v) in buffered saline containing 0.15 mM $^{45}Ca^{2+}$ (0.8 μCi/ml), and the amino acid mixture were incubated with 4 μmol A23187/kg cells at 39°C. At 30 min, 10 μmol A23187/kg cells were added and 1.5 min later additions were made (⟶) of lipid (PC, ●) or MgEGTA, 1 mM final conc. (EMg, ▲) or buffered saline (○, △). All additions were 0.020 ml/cell suspension. Aliquots of 0.50 ml were mixed at the indicated times with 9.0 ml ice-cold diluent (149 mM NaCl, 0.033 mM MgEGTA, 5 mM TES (pH 7.4) and 0.05 mM [^{3}H]maltitol, 0.04 μCi/ml) and promptly centrifuged in a Sorval RC2-B centrifuge 14600 × *g* for 5 min.

After lipid treatment, the residual influx was 7 μmol . kg^{-1} . min^{-1} above the influx of cells never exposed to A23187.

Asolectin (soybean 'lecithin') also works but it contains Ca^{2+} (2.3 nmol/g) and binds considerable Ca^{2+}, as measured by atomic absorption and dialysis, respectively. The egg lipid had 0.73 mmol Ca^{2+}/g and undetectable Ca^{2+} binding.

Fig.2 shows the cells' responses to a 1.5 min $^{45}Ca^{2+}$ influx pulse (apparent influx: 38 μmol . kg^{-1} . min^{-1}) started by A23187 addition and stopped by addition of either lipid or MgEGTA.

In this experiment, the cells' exchangeable Ca^{2+} pool(s) was first labeled with $^{45}Ca^{2+}$ using 4 μmol A23187/kg cells. This low dose simply labels the cell pool(s) without enlarging it (unpublished). The influx pulse was started by adding 10 μmol A23187/kg cells (apparent influx $\propto$ $[A23187]^{2.9}$, unpublished). Whether $^{45}Ca^{2+}$ influx was stopped by lipid or by MgEGTA, the resulting initial $^{45}Ca^{2+}$ effluxes were similar. However, during the 1.5 min exposure to high A23187, some $^{45}Ca^{2+}$ entered a cell compartment from which it was not expelled by the Ca^{2+} pump after lipid addition.

In this experiment, cells were very quickly brought to a non-equilibrium, non-steady state with respect to Ca^{2+}, and quickly released to 'decay' back toward their original condition. So far as we are aware, it can be done by this procedure only.

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