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Protein kinase C dependent and independent activation of phospholipase A_2 under calcium ionophore (A23187) exposure in rabbit pulmonary arterial smooth muscle cells

Sajal Chakraborti[1], John R. Michael[2] and Samir K. Patra[1]

[1] *Department of Biochemistry and Biophysics, University of Kalyani, Kalyani 741 235, India* and [2] *Department of Medicine, University of Utah Health Sciences Center, Salt Lake City, Utah 84132, USA*

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Exposure of rabbit pulmonary arterial smooth muscle cells to the calcium ionophore A23187, dose-dependently stimulates arachidonic acid (AA) release and phospholipase A_2 (PLA_2) activity. The protein kinase C (PKC) inhibitor, sphingosine does not prevents AA release and PLA_2 activity caused by low doses of A23187. In contrast, sphingosine markedly prevents AA release and PLA_2 activity caused by higher doses of A23187. PKC activity profile indicates that treatment of the cells with low doses of A23187 does not cause significant alteration of PKC translocation from cytosol to membrane whereas higher concentrations of the ionophore dose-dependently enhance PKC translocation from cytosol to membrane in the smooth muscle cells.

Calcium ionophore; Phospholipase A_2; Arachidonic acid; Sphingosine; Protein kinase C; Smooth muscle cell; Calcium signalling

1. INTRODUCTION

The calcium ionophore, A23187 is a widely accepted agent used as a probe to study the calcium signalling phenomena in cells and tissues. A23187 was shown to stimulate release of AA and its metabolites in the presence of extracellular calcium in various types of cells [1–3]. Studies on different types of cells indicated that protein kinase C activators potentiate the effect of calcium ionophore to release AA and its metabolites [4–6]. One of the steps that would require the participation of protein kinase C might occur at the level at which AA is released from its phospholipid stores upon stimulation of PLA_2 [4], a calcium activated enzyme [7]. In view of this, and to gain insight into the calcium signalling phenomena caused by a calcium ionophore, A23187 was used to stimulate AA release and PLA_2 activity, and the precise role of protein kinase C in this scenario in rabbit pulmonary arterial smooth muscle cells is determined in this present investigation.

Abbreviations: AA, arachidonic acid; PLA_2, phospholipase A_2; A23187, calcium ionophore; PKC, protein kinase C; PBS, phosphate buffered saline; FCS, fetal calf serum; HEPES, 4-(2-hydroxyethyl)-1-piperazine *N*-2-ethanesulfonate; ATP, adenosine triphosphate; HBPS, Hank's buffered physiological solution.

Correspondence address: S. Chakraborti, Department of Biochemistry and Biophysics, University of Kalyani, Kalyani 741 235, India.

2. MATERIALS AND METHODS

2.1. *Reagents*

HBPS, DMEM, FCS, PBS without Ca^{2+} and Mg^{2+}, and trypsin were purchased from GIBCO laboratories (Grand Island, NY). Sphingosine, HEPES, calcium ionophore (A23187), fatty-acid-free bovine serum albumin, dithiothreitol, ATP, leupeptin, histone type IIIs, collagenase, and type I porcine pancreatic elastase were obtained from Sigma Chemical Co., St. Louis, MO. [^{14}C]Arachidonic acid, the radiolabelled phospholipid L-3-phosphatidyl choline, L-1-stearoyl-2 [1-^{14}C]arachidonyl and [γ-^{32}P]ATP were obtained from New England Nuclear, Wilmington, DE. All other chemicals used were of analytical grade.

2.2. *Cell culture*

The pulmonary arterial smooth muscle cells of rabbit were isolated by following the method of Ross [8] with some modifications. Briefly, the main pulmonary artery and its bifurcation were isolated and placed in HBPS to remove the blood cells. The artery was incubated at 37°C in a collagenase and elastase mixture (1 mg/ml of collagenase plus 0.25 mg/ml of type I porcine pancreatic elastase). After incubation for 30 min, the tissue was minced into 1–3 mm^2 pieces with scalpel blades. The tissue was incubated for an additional two hours with fresh enzyme added every 30 min. The solution was filtered through sterile 100 μ mesh and centrifuged at 100 × *g* for 5 min at 4°C. The cells were then placed in DMEM with 20% fetal calf serum, 100 units of streptomycin and penicillin, L-glutamine, non-essential amino acids and 1.5 g sodium bicarbonate per liter. Medium was changed 3 times per week. Cells were subcultured after treatment with 0.25% trypsin. Cells were studied between passages 7 and 12. All experiments were performed on confluent monolayers and in serum-free media supplemented with fatty-acid-free bovine serum albumin (1 mg/ml).

2.3. *Measurements of [^{14}C]AA release and PLA_2* activity

Cells grown in six-well plates (Costar) were washed twice with PBS

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(pH 7.2) and incubated for 20 h with [^{14}C]arachidonic acid (sp. activity 54.6 mCi/mmol; 2 μCi/well). After incubation, the supernatant was removed and the cells were washed twice with PBS. To measure A23187-induced AA release, cells were exposed to different concentrations of A23187 for 10 min. The medium was then removed and AA release was measured following the method of Chakraborti et al. [9]. To determine A23187-induced PLA_2 activity, the smooth muscle cells grown in six-well plates (Costar) were incubated with different concentrations of the ionophore A23187, for 10 min. After incubation, the monolayers were washed twice with PBS (pH 7.2). The cells were then removed from PBS with a rubber policeman and centrifuged in a microfuge at 12 000 × g for 2 min at room temperature. The pellet was suspended in PBS and disrupted by sonication in a cell sonifier. Twenty microliters of the broken cell preparation (1–3 mg protein ~ 10^5 cells) were added to 30 μl of the reaction mixture, which contained (final concentrations): Tris-hydroxymethylaminomethane (Tris) buffer (100 μM), NaCl (100 mM), deoxycholate (1 mM) and the phospholipid L-3-phosphatidyl choline-L-1-stearoyl-2-[1-^{14}C]arachidonyl (sp. activity 58.3 mCi/mmol; 10 μM). Phospholipase A_2 activity was assayed at pH 9.0 following the method of Chakraborti et al. [9]. To determine the optimum concentration of sphingosine to inhibit stimulation of AA release caused by A23187, cells were pretreated with different concentrations of sphingosine for 20 min before addition of 10 μM of the ionophore for 10 min, and AA release was measured. To determine the effect of sphingosine on A23187-induced AA release and PLA_2 activity, the cells were pretreated with sphingosine (15 μM) for 20 min before exposure to different concentrations of the ionophore for 10 min, and AA release and PLA_2 activity were measured.

2.4. *Measurement of protein kinase C activity*

The smooth muscle cells grown in T-150 flasks were washed twice with PBS (pH 7.2) and incubated in DMEM supplemented with 1 mg/ml fatty-acid-free bovine serum albumin for 10 min. The medium was then removed and the cells washed with ice-cold homogenizing medium (containing 20 mM HEPES-NaOH (pH 7.4), 5 mM EGTA, 3 mM $MgCl_2$, 0.1% fatty-acid-free bovine serum albumin). The cells were then scraped from the flasks and disrupted by sonication with a cell sonifier and centrifuged at 100 000 × g for 1 h at 4°C. The protein concentration of the supernatant fraction was maintained at 1 mg/ml with the homogenizing medium and this fraction was used as the cytosolic fraction. The pellet was then resuspended in ice-cold homogenizing buffer containing 0.1% Triton X-100. After sonication, the pellet was kept at 4°C with constant shaking for 1 h and then centrifuged at 100 000 × g for 1 h at 4°C. The supernatant fraction was used as the membrane fraction and protein concentration was maintained at 1 mg/ml with the homogenizing medium that was supplemented with Triton X-100 (0.1%). Protein kinase C activity was determined following the method of Kitano et al. [10] with some modifications. Briefly, a 20 ml aliquot of the sample of the cytosolic, or membrane fraction was added to 80 μl of the reaction mixture containing 25 mM HEPES-NaOH (pH 7.4), 2 mM DTT, 20 μM ATP, 0.2 mg/ml histone type IIIs, 10 μM [γ-^{32}P]ATP (50–100 cpm/pmol) with or without 1.5 mM $CaCl_2$, 25 μg/ml phosphatidyl serine, and 0.5 μg/ml diolein. Incubation was performed at 30°C for 10 min. The reaction was terminated with the addition of 1 ml of ice-cold stopping solution containing 10% TCA and 2 mM ATP, followed by the addition of 100 μl of 0.5% fatty-acid-free bovine serum albumin. The precipitated protein was trapped on a millipore HA filter. The filter was washed 5 times, each time with 3 ml of cold 5% TCA, and dried. The radioactivity remaining on the filter was determined by subtracting the activity measured in the absence of calcium and the phospholipid from that measured in their presence for both cytosol and membrane fractions.

To determine the effect of different concentrations of A23187 on protein kinase C activity, the smooth muscle cells were treated with different concentrations of the ionophore for 10 min, and protein kinase C activity in the cytosol and membrane fractions was determined.

2.5. *Cell viability*

None of the agents used in this present study affected the cell viability as assessed by Trypan blue exclusion.

2.6. *Statistical analysis*

Data were analyzed by unpaired *t*-test, repeated measures analysis of variance, and the Duncan multiple range test for group comparisons. Statistical significance was assumed when $P<0.05$ [11].

3. RESULTS AND DISCUSSION

The calcium ionophore, A23187 dose-dependently stimulates AA release and PLA_2 activity in rabbit pulmonary arterial smooth muscle cells (Fig. 1). The stimulation of AA release and PLA_2 activity were determined to be optimum at 10 μM of the ionophore (A23187) treatment to the cells (Fig. 1). This implies that the induction of PLA_2 activity resulting in the stimulation of AA release must require a rise in intracellular calcium concentration, and that a calcium-sensitive component could be involved in the calcium ionophore-mediated stimulation of AA release and PLA_2 activity in the smooth muscle cells. The requirement of protein kinase C for stimulation of AA release and PLA_2 activity in the cells under A23187 exposure is determined by a protein kinase C inhibitor, sphingosine. Sphingosine was demonstrated to inhibit protein kinase C activity in in vitro systems [12,13]. The optimum concentration (10 μM) of sphingosine required to produce its maximum inhibitory effect on A23187-induced AA release was determined to be of 15 μM (Fig. 2). Sphingosine does not prevent AA release and PLA_2 activity caused by low doses of A23187 (Fig. 1). In contrast, the stimulation of AA release and PLA_2 activity caused by higher doses of A23187 was determined to be markedly prevented by sphingosine (Fig. 1). It also appears from the present study that treatment of the cells with low doses of A23187 does not significantly stimulate protein kinase C translocation from cytosol to membrane (Table I), whereas higher doses of the ionophore dose-dependently stimulate protein kinase C translocation from cytosol to membrane (Table I). Overall, from the present observations we conclude that there are 2 distinct mechanisms for activation of PLA_2 activity which occur under A23187 exposure in pulmonary arterial smooth muscle cells. The potent one occurs under higher doses of A23187 treatment with the involvement of protein kinase C, and the minor contribution to a marginal activation of PLA_2 happens as a result of a small rise in intracellular calcium caused by low doses of the ionophore treatment and does not occur through the involvement of protein kinase C dependent mechanism(s). Protein kinase C may stimulate AA release and PLA_2 activity by several possible mechanisms including activation of PLA_2 by phosphorylation [14], regulation of PLA_2 stimulatory proteins [15], or regulation of PLA_2 inhibitory proteins such as lipocortins [16].

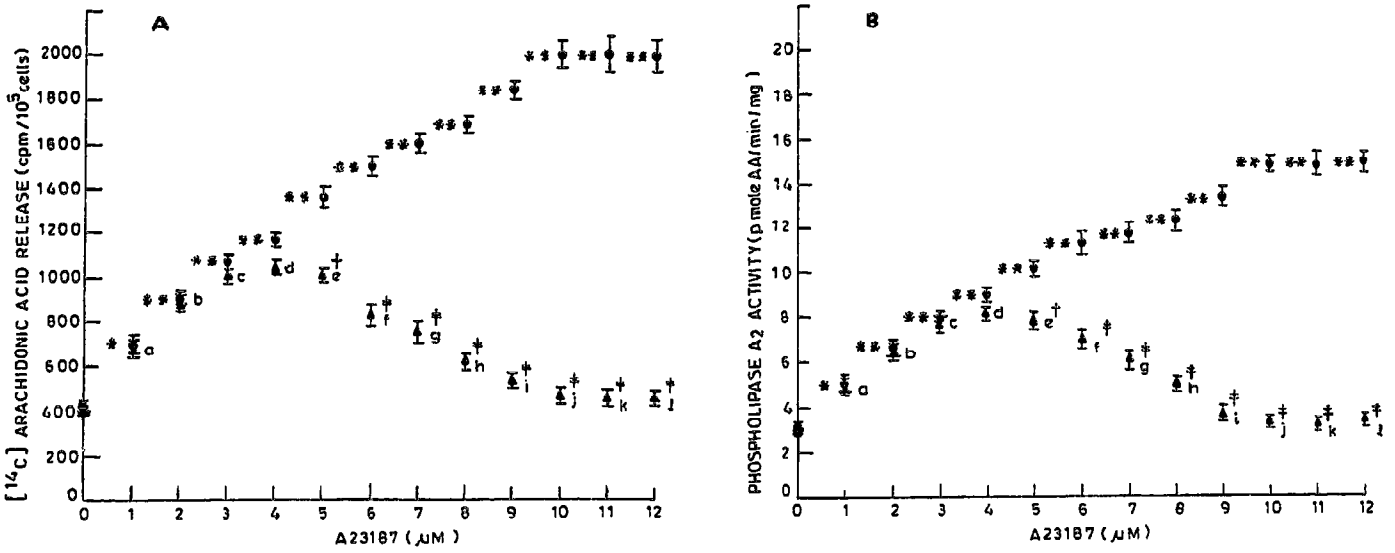

Fig. 1. Effect of sphingosine on AA release (A) and PLA_2 activity (B) caused by different concentrations of calcium ionophore (A23187) in rabbit pulmonary arterial smooth muscle cells. •, Calcium ionophore (A23187)-mediated AA release (A), and PLA_2 activity (B); ▲, Sphingosine (15 μM)-induced effect on calcium ionophore (A23187)-mediated AA release (A), and PLA_2 activity (B). Results are means ± SE ($n = 4$); *$P < 0.05$ compared to control; **$P < 0.001$ compared to control; †$P < 0.05$ compared to respective treatment A23187; ‡$P < 0.001$ compared to respective treatment A23187. Percent decrease in (A) AA release by sphingosine (15 μM) over the response produced by different concentrations of calcium ionophore (A23187): a, 7%; b, 6%; c, 10%; d, 19%; e, 38%; f, 61%; g, 72%; h, 84%; i, 92%; j, 96%; k, 96%; l, 96%. (Percent change in AA release caused by sphingosine (15 μM) over control, 2%.) [AA release (cpm/10^5 cells) produced: control, 410 ± 42; sphingosine, 403 ± 38.] (B) PLA_2 activity by sphingosine (15 μM) over the response produced by different concentrations of calcium ionophore (A23187): a, 10%; b, 9%; c, 10%; d, 11%; e, 31%; f, 52%; g, 65%; h, 78%; i, 93%; j, 98%; k, 98%; l, 98%. (Percent change in PLA_2 activity caused by sphingosine (15 μM) over control, 3%.) [PLA_2 activity (pmol AA/min/mg) produced: control, 3.12 ± 0.28; sphingosine, 3.01 ± 0.32.]

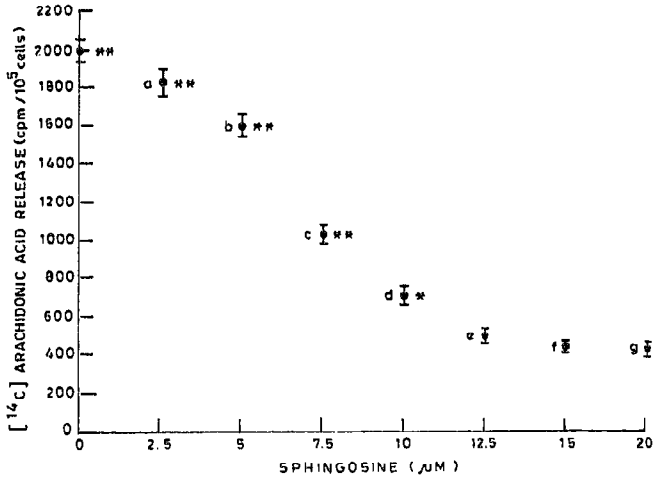

Fig. 2. Dose-response profile of sphingosine to prevent A23187-mediated AA release in rabbit pulmonary arterial smooth muscle cells. Results are mean ± SE ($n = 4$); *$P < 0.05$ compared to control; **$P < 0.001$ compared to control. [AA release (cpm/10^5 cells) produced: control, 410 ± 42 vs 10 μM of A23187, 1995 ± 128.] Percent decrease in AA release produced by different concentration of sphingosine over the response produced by 10 μM of A23187: a, 11%; b, 25%; c, 61%; d, 82%; e, 94%; f, 98%; g, 98%.

Table I

Effect of different concentrations of the calcium ionophore (A23187) on the subcellular distribution of protein kinase C in rabbit pulmonary arterial smooth muscle cells

Treatments	Protein kinase C activity (pmol/min/mg)		
	Cytosol	Membrane	Total
None	1725 ± 102 (90)	184 ± 34 (10)	1909
A23187 (1 μM)	1718 ± 92 (91)	178 ± 26 (9)	1896
A23187 (2 μM)	1712 ± 98 (90)	186 ± 32 (10)	1898
A23187 (3 μM)	1706 ± 88 (90)	192 ± 24 (10)	1898
A23187 (4 μM)	1563 ± 74* (81)	362 ± 38* (19)	1925
A23187 (5 μM	1328 ± 78** (69)	594 ± 45** (31)	1922
A23187 (6 μM)	1196 ± 66** (62)	719 ± 44** (38)	1915
A23187 (7 μM)	1092 ± 42** (57)	816 ± 55** (43)	1908
A23187 (8 μM)	779 ± 38** (40)	1145 ± 38** (60)	1924
A23187 (9 μM)	520 ± 26** (27)	1398 ± 66** (73)	1918
A23187 (10 μM)	349 ± 32** (18)	1563 ± 78** (82)	1912
A23187 (11 μM)	356 ± 26** (18)	1576 ± 56** (82)	1932
A23187 (12 μM)	362 ± 35** (19)	1552 ± 64** (81)	1914

Results are mean ± SE ($n = 4$). Numbers in the parentheses represent percentages of protein kinase C in cytosol and membrane fractions of the smooth muscle cells.

*$P < 0.05$ compared to control; **$P < 0.001$ compared to control

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