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A23187 causes release of inositol phosphates from cultured rat Kupffer cells

Chandrashekhar R. Gandhi [a,*], Stephen A.K. Harvey [b], Margot Cevallos [b], Merle S. Olson [b]

[a] *Departments of Surgery and Pathology, Thomas E. Starzl Transplantation Institute, University of Pittsburgh, E-1540 BST, 200 Lothrop Street, Pittsburgh, PA 15213, USA*

[b] *Department of Biochemistry, University of Texas Health Science Center, San Antonio, TX 78284-7760, USA*

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Abstract

The Ca^{2+} ionophore A23187 is routinely used to illustrate the extracellular Ca^{2+}-dependence of a variety of cellular reactions. We found that A23187-induced hydrolysis of phosphoinositides to various inositol phosphates in rat Kupffer cells was accompanied by their release from the cells. The synthesis and release of inositol phosphates was A23187 concentration-dependent (0.5–10 μM), and was apparent at the lowest concentration tested. A23187-induced release of inositol phosphates increased time-dependently, was apparent at 5 s of stimulation and maximal at 20 min. The effects of A23187 were reversed by EGTA. The integrity of the cells was not affected by A23187 treatment as indicated by their exclusion of trypan blue and the lack of release of lactate dehydrogenase. We propose that such effects should be considered while evaluating the Ca^{2+}-dependence of biological processes based on the actions of A23187. © 2001 Published by Elsevier Science B.V.

Keywords: A23187; Ca^{2+} ionophore; Kupffer cell; Inositol phosphate

1. Introduction

The biochemical effects of the Ca^{2+} ionophore A23187 on rat liver mitochondria were reported more than 25 years ago (Reed and Lardy, 1972a,b). Since then A23187 has been used to provide a "positive control" for extracellular Ca^{2+} ($[Ca^{2+}]_e$) dependence of a variety of cellular processes. Recently, A23187 has also been used to demonstrate Ca^{2+}-dependence of such processes as nitric oxide synthesis (Shinozaki et al., 1999) and apoptosis (Lemasters, 1999). In several cell types, A23187 has been shown to stimulate hydrolysis of phosphoinositides into soluble second messengers inositol phosphates (Brammer et al., 1988; Emilsson and Sundler, 1984; Moscat et al., 1988; Williamson et al., 1985). While investigating phosphoinositide metabolism and changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) induced by platelet activating factor and other agonists in hepatic Kupffer cells, we noticed an unusual A23187-mediated cellular formation and accumulation of inositol phosphates. This observation prompted us to investigate more closely its effects on phosphoinositide metabolism in these cells. The present experiments demonstrate that although A23187 stimulates the metabolism of phosphoinositides to inositol phosphates, a major portion of these highly charged metabolites are released into the extracellular medium. This phenomenon does not appear to be a consequence of cell injury elicited by increased $[Ca^{2+}]_i$ since the cells treated with A23187 did not permeate trypan blue or release lactate dehydrogenase into the culture medium.

2. Materials and methods

The following reagents and chemicals were purchased from the indicated sources: William's medium E and antibiotics (GibcoBRL, Grand Island, NY, USA); collagenase (Type IV), protease (Type XIV), bovine serum albumin (fraction V) and EGTA (Sigma, St. Louis, MO, USA);

* Corresponding author. Tel.: +1-412-648-9316; fax: +1-412-624-6666.

E-mail address: GANDHICS@MSX.UPMC.EDU (C.R. Gandhi).

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myo-2[^{3}H]inositol (20 Ci/mmol) and mixed [^{3}H]inositol phosphate standards (Amersham, Arlington Heights, IL); synthetic platelet activating factor (1-*O*-hexadecyl-2-*O*-acetyl-sn-glycero-3-phosphocholine) (Bachem, Bubendorf, Switzerland); metrizamide (2-(3-acetamido-5-*N*-methyl-acetamido-2,4,6-triiodobenzamido)-2-deoxy-D-glucose) (Nyegaard, Oslo, Norway); and A23187 (Calbiochem, San Diego, CA, USA).

2.1. Isolation and culture of Kupffer cells

Kupffer cells were isolated from the livers of male Sprague–Dawley rats (200–250 g) as described previously (Gandhi et al., 1990). Briefly, following collagenase and protease digestion of the liver, nonparenchymal cells were separated from hepatocytes and cell debris by low speed centrifugation, and purified on a metrizamide gradient. Kupffer cells were then purified from other nonparenchymal cells by centrifugal elutriation. The cells were suspended in William's medium E supplemented with 2 mM L-glutamine, 10% fetal calf serum and penicillin/streptomycin, and placed at a density of 4×10^6 cells in 35 mm tissue culture dishes. The medium was renewed following an overnight incubation in a humidified atmosphere of air/CO_2 (95:5). Cells were used for experiments on day 3 or 4 of culture.

2.2. Metabolism of phosphoinositides

Cells were washed and placed in fresh medium containing 2% serum and 2 μCi/ml [^{3}H]inositol. After a 24-h labeling period, cells were washed with serum-free medium without [^{3}H]inositol containing 0.25% bovine serum albumin, and 10 mM lithium chloride to inhibit the breakdown of inositol phosphates (Dean and Moyer, 1987; Downes and Michell, 1981). Cells were challenged with specified concentrations of the agents as indicated in the figure and table legends. After various experimental treatments, the medium was aspirated, centrifuged and the supernatant stored at −80°C in polypropylene tubes. Cell extracts were prepared by replacing the medium with 1 ml of 10% (w/v) ice-cold trichloroacetic acid. After scraping and centrifugation for 5 min at $14{,}000 \times g$, the supernatant was removed, washed five times with a fivefold excess of water-saturated ether, lyophilized and stored at −80°C.

Inositol phosphates were analyzed by anion exchange chromatography on Dowex column (Downes and Michell, 1981). Briefly, the medium or the cell extract reconstituted in water was loaded on to a column of 1 g Dowex-1X (200–400 mesh size; Sigma) washed successively with 2 N HCl, water, 3 M ammonium formate and water. The column was washed with 30 ml water, and [^{3}H]glycerophosphoinositol, [^{3}H]inositol monophosphate, [^{3}H]inositol bisphosphate and [^{3}H]inositol trisphosphate were eluted, respectively, with 10 ml of 60 mM sodium borate/5 mM sodium tetraborate, 0.2 M ammonium formate in 0.1 M formic acid, 0.4 M ammonium formate in 0.1 M formic acid and 1 M ammonium formate in 0.1 M formic acid. Fractions were collected, and radioactivity was determined in a Beckman LS5801 β counter at about 50% efficiency for ^{3}H. The identity of individual inositol phosphates was determined by an HPLC procedure essentially as described previously (Gandhi et al., 1990).

2.3. Determination of cell membrane integrity

2.3.1. Lactate dehydrogenase assay

Lactate dehydrogenase activity in the medium of cultured cells was determined essentially as described by Wroblewski and LaDue (1953). Cells were washed and placed in Hank's balanced salt solution containing 0.1% bovine serum albumin and challenged with platelet activating factor or A23187 for 20 min. For complete release of lactate dehydrogenase activity, cells were incubated with 0.2% (final concentration) Triton X-100. The assay mixture contained 2.4 ml of 0.05 M phosphate buffer, pH 7.4, 0.1 ml incubation medium, and 0.1 ml (2.5 mg) NADH. After 20 min, 0.1 ml (2.5 mg) sodium pyruvate was added and the absorbance was recorded for 5 min at 340 nm.

2.3.2. Trypan blue exclusion

For determination of trypan blue exclusion, cells were treated with trypsin (0.25% in Hank's balanced salt solution containing 1 mM EDTA; GibcoBRL) following incubation with A23187, suspended in the culture medium and mixed with trypan blue (0.4%; Sigma) (1:1, v/v). Viability of the cell was then determined in a hemocytometer.

2.3.2.1. Data analysis. The values are presented as averages of triplicate determinations. Each experiment was repeated at least three times using separate cell preparations. Student's *t*-test was employed for statistical comparison of the paired samples. A P value of < 0.05 was considering statistically significant.

3. Results

3.1. Effect of A23187 and platelet activating factor on phosphoinositide metabolism in Kupffer cells

Both platelet activating factor and A23187 caused significant increase in the synthesis of [^{3}H]glycerophosphoinositol and [^{3}H]inositol phosphates in the cells (Fig. 1). These results suggest activation of phospholipase A (formation of glycerophosphoinositol) as well as phospholipase C (formation of inositol phosphates) (Emilsson and Sundler, 1984; Williamson et al., 1985) by both agents. However, stimulation with platelet activating factor evoked a much greater elevation of these metabolites compared to that with A23187. Upon analysis of the culture media from cells incubated with these agents, release of [^{3}H] glycero-

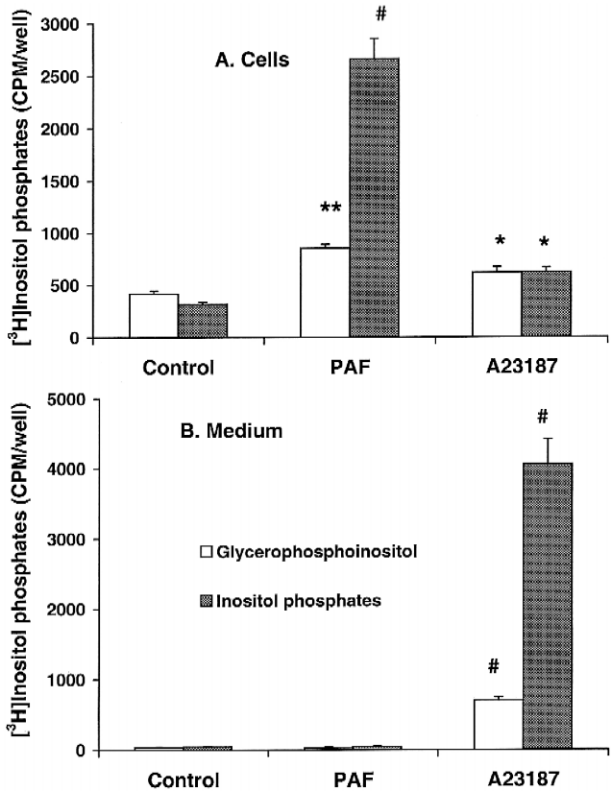

Fig. 1. Effect of A23187 on the formation and the release of [^{3}H]inositol phosphates. [^{3}H]inositol-labeled Kupffer cells were stimulated with 25 nM platelet-activating factor (PAF) or 10 μM A23187 for 20 min. Cell extracts (A) and the medium (B) were analyzed for [^{3}H]glycerophosphoinositol and [^{3}H]inositol phosphates by Dowex chromatography. $^{*}P < 0.05$; $^{**}P < 0.01$; $\#P < 0.001$ vs. control.

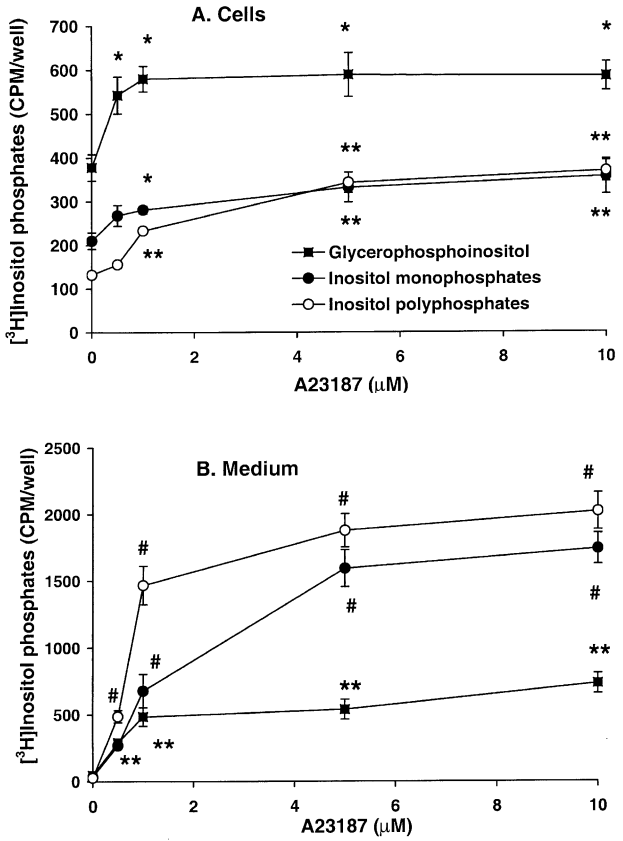

Fig. 2. Concentration dependence of A23187-mediated formation and release of inositol phosphates. [^{3}H]inositol-labeled cells were stimulated with indicated concentrations of A23187 for 20 min. Radioactivity associated with [^{3}H]inositol phosphates in the cells (A) and the medium (B) was determined by Dowex column chromatography. $^{*}P < 0.05$; $^{**}P < 0.01$; $\#P < 0.001$ vs. control.

phosphoinositol and [^{3}H]inositol phosphates was observed only from A23187-challenged cells.

3.2. Concentration dependence and time course of A23187-mediated formation and release of inositol phosphates

A23187-induced cellular accumulation (Fig. 2A) and extracellular release (Fig. 2B) of [^{3}H]inositol phosphates increased in a concentration-dependent manner starting at the concentration of A23187 as low as 0.5 μM. Up to a concentration of 1 μM, A23187-induced release of [^{3}H]inositol monophosphates and [^{3}H] glycerophosphoinositol into the extracellular medium was parallel. Thereafter, the accumulation of [^{3}H]inositol monophosphates into the medium became progressively much higher with a maximal effect occurring at 5 μM A23187. On the other hand, the release of [^{3}H] glycerophosphoinositol into the medium remained constant. Also, A23187-induced release of [^{3}H]inositol polyphosphates increased in a concentration-dependent fashion. However, the release of these metabolites into the medium exceeded their accumulation in cells at all concentrations of A23187 tested.

A23187-dependent release of [^{3}H]inositol phosphates from Kupffer cells was evident at 1 min of stimulation, and was maximal at about 20 min of stimulation (Fig. 3).

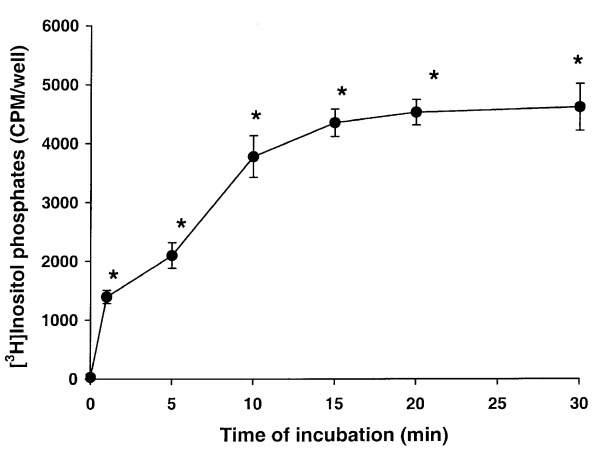

Fig. 3. Time course of A23187-mediated release of inositol phosphates. [^{3}H]inositol-labeled cells were stimulated with 10 μM A23187. At the indicated time points, the reaction was terminated and [^{3}H]inositol phosphates in the medium was analyzed. $^{*}P < 0.001$ vs. control.

3.3. Effect of EGTA on A23187-mediated release of inositol phosphates

In the experiment illustrated in Fig. 4, [^{3}H]inositol-labeled Kupffer cells were challenged with platelet activating factor, A23187 and EGTA + A23187 for 5 s and 20 min. At 5 s, the levels of [^{3}H]inositol bis- and trisphosphates increased in PAF-stimulated, but not in A23187-stimulated cells (Fig. 4A). As determined by HPLC, these metabolites were identified as [^{3}H]inositol-1,4-bisphosphate and [^{3}H]inositol-1,4,5-trisphosphate, respectively. However, even at this early time point of A23187 stimulation [^{3}H]inositol mono- (identified as [^{3}H]inositol-1-phosphate by HPLC) and polyphosphates (identified as [^{3}H]inositol-1,4-bisphosphate and [^{3}H]inositol-1,4,5-triisphosphate by HPLC) were released into the medium (Fig. 4B). At 20 min of stimulation, small increases in various [^{3}H]inositol phosphates were observed in A23187-stimulated cells (Fig. 4C) since major portions of these metabolites were released into the medium (Fig. 4D). HPLC determination revealed that [^{3}H]inositol monophosphate consisted of 80% [^{3}H]inositol-1-phosphate and 20% [^{3}H]inositol-4-phosphate. [^{3}H]inositol-1,4-bisphosphate formed more than 90% of the total [^{3}H]inositol bisphosphate with the rest being [^{3}H]inositol-1,3-bisphosphate. [^{3}H]inositol trisphosphate consisted of approximately 80% [^{3}H]inositol-1-4-5, trisphosphate and about 20% [^{3}H]inositol-1,3,4-trisphosphate. The presence of EGTA in the incubation medium prevented the cellular synthesis as well as the extracellular release of inositol phosphates in A23187-stimulated cells (Fig. 4B–D). These results indicate a requirement for extracellular Ca^{2+} for A23187-induced stimulation of phosphoinositide metabolism.

3.4. Effect of A23187 on the membrane integrity

In order to elucidate whether the A23187-mediated release of inositol phosphates was a consequence of cell injury or increased membrane permeability induced by the

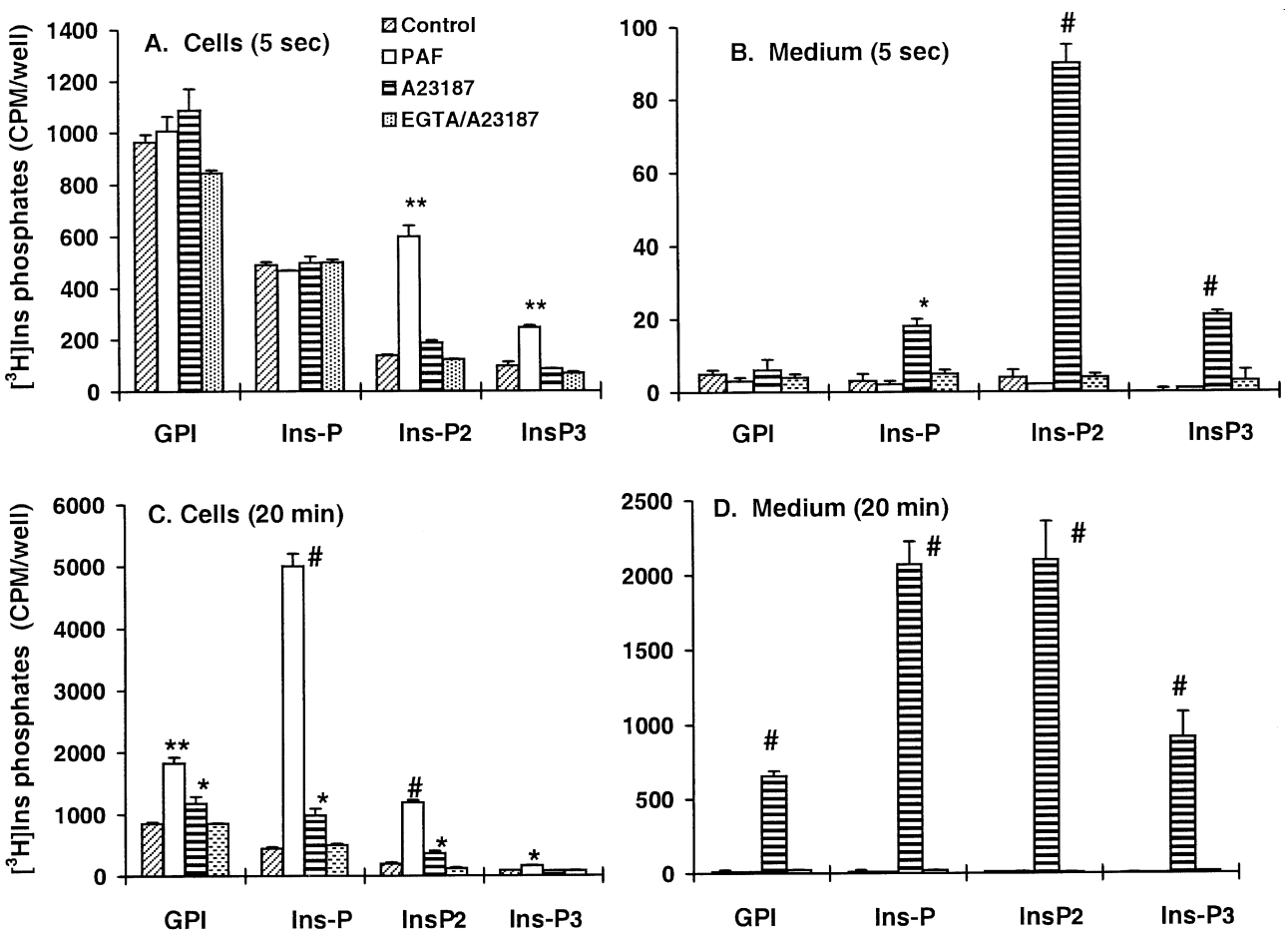

Fig. 4. Effect of EGTA on A23187-induced formation and release of inositol phosphates at 5 sec and 20 min. Cells labeled with 5 μCi/ml [^{3}H]inositol were challenged with 25 nM platelet activating factor (PAF) or 10 μM A23187 ± 5 mM EGTA. Upper panels (A and B) show [^{3}H]inositol phosphates in the cells and the medium as determined by Dowex column chromatography at 5 s of stimulation, whereas the lower panels (C and D) illustrate [^{3}H]inositol phosphates at 20 min. $^{*}P < 0.05$; $^{**}P < 0.01$; $\#P < 0.001$ vs. control. GPI, glycerophosphoinositol; Ins-P, inositol monophosphates; Ins-P_2, inositol bisphosphates; Ins-P_3, inositol trisphosphates.

Table 1
Effect of A23187 on the release of lactate dehydrogenase from Kupffer cells

	Decrease in A_{340} over 5 min	% LDH activity
Triton X-100 (0.2%)	0.298 ± 0.01	100 ± 4
Vehicle	0.019 ± 0.004	6.4 ± 1.3
A23187 (10 μM)	0.020 ± 0.005	6.7 ± 1.8
PAF (50 nM)	0.017 ± 0.003	5.6 ± 0.9

Cells were washed and incubated with indicated concentrations of A23187, platelet activating factor (PAF) or Triton X-100 for 20 min. Control cells were incubated with the respective vehicles (dimethyl sulfoxide for A23187 and 0.2% bovine serum albumin in 0.9% NaCl for PAF). Lactate dehydrogenase (LDH) activity in the supernatant medium was determined as described in the Methods section.

ionophore, the effect of A23187 on the release of lactate dehydrogenase by Kupffer cells was examined. The release of lactate dehydrogenase from cells incubated with Triton X-100, used as a positive control, was considered to be 100%. Neither A23187 (10 μM) nor platelet activating factor (50 nM) caused an appreciable increase in the lactate dehydrogenase activity in the medium over the basal level (Table 1). Furthermore, at the concentrations of A23187 used, as determined by the trypan blue exclusion criteria in Kupffer cells, the integrity of the plasma membrane was not affected (results not shown).

Similar effects of A23187 were also noted in rat cardiomyocytes as well as with another Ca^{2+} ionophore ionomycin in Kupffer cells (results not shown).

4. Discussion

The present study demonstrates that treatment of Kupffer cells with a widely used Ca^{2+} ionophore A23187 results in the stimulation of phosphoinositide metabolism and the release of inositol phosphates into the extracellular medium. This unique effect of A23187 may not be a consequence of generalized increased membrane permeability or cell injury since A23187-challenged cells excluded trypan blue and did not release lactate dehydrogenase. Earlier work demonstrated A23187 stimulated formation of inositol phosphates in many cell types including macrophages (Emilsson and Sundler, 1984), vascular endothelial cells (Moscat et al., 1988) and neuronal cells (Brammer et al., 1988). Interestingly, however, A23187 was found not to stimulate phosphoinositide hydrolysis in certain cell types such as hepatocytes (Creba et al., 1983; Thomas et al., 1983, 1984) and platelets (Moscat et al., 1986; Rittenhouse, 1984). Equivalent formation of inositol phosphates was reported in cultured aortic endothelial cells challenged with endothelial cell growth factor (1 ng/ml) and A23187 (10 μg/ml) (Moscat et al., 1988). However, in these experiments, combined concentration of inositol phosphates in cells and the medium appears to have been determined. Therefore, it is difficult to assess whether these cells also released inositol phosphates upon A23187 stimulation. It is also found that thrombin-induced metabolism of phosphoinositides in platelets is $[Ca^{2+}]_e$-independent, but when platelets were pretreated with A23187 the effect of thrombin was abolished (Moscat et al., 1986). In contrast, pretreatment with A23187 did not affect subsequent vasopressin-induced phosphoinositide metabolism in hepatocytes (Thomas et al., 1983). Interestingly, thrombin-induced release of 5-hydroxytryptamine from platelets was unaffected by A23187 pretreatment (Moscat et al., 1986). Although these results indicate that the release of 5-hydroxytryptamine is Ca^{2+}-independent, in consideration of the observations of the present study, it is quite likely that inositol phosphates formed in response to thrombin (Moscat et al., 1986) may have leaked out from A23187-treated platelets.

Increased $[Ca^{2+}]$i, due to its influx across the plasma membrane induced by A23187, has been suggested to stimulate secretion of hormones and neurotransmitters (Pressman and Fahim, 1982). However, high concentrations (5–10 μM) of ionophore must be used in order to induce effects on secretion (Knight and Baker, 1983; Tam and Dannies, 1980). Knight and Baker (1983) reported that A23187 caused the release of catecholamines from isolated adrenal medullary cells, and unlike other mediators where the response was reproducible, the A23187-induced release of catecholamines was inconsistent and varied between 0 and 70% of the total cellular catecholamines. Furthermore, higher rates of catecholamine release were associated with large amounts of cell debris and the permeability of a large proportion of the cells to trypan blue (Tam and Dannies, 1980). These findings are consistent with the notion that cellular injury and death can involve an increase in the cytosolic Ca^{2+} concentration. In this regard, low density lipoprotein-induced increases in $[Ca^{2+}]_i$ in lymphoblastoid cells has been suggested to cause DNA fragmentation and apoptosis (Escargueil et al., 1992). A23187 has also been shown to cause apoptosis of various cells (Lemasters, 1999). However, the concentration of the ionophore used is generally high (5–10 μM) and the time of incubation much longer (several minutes to hours) (Lemasters, 1999). In contrast to these findings, cellular formation and extracellular release of inositol phosphates in Kupffer cells occurred at low (e.g., 0.5 μM) concentrations of A23187 and at early time points (e.g., 5 s). Furthermore, the cells were not damaged as indicated by their lack of permeability to trypan blue and their failure to release lactate dehydrogenase.

The formation of inositol phosphates induced by A23187 appears to be dependent upon extracellular Ca^{2+} as the presence of the Ca^{2+} chelator, EGTA, completely abrogated this processes. Agonists known to increase $[Ca^{2+}]_i$ and stimulate phosphoinositide metabolism do not in themselves appear to cause the release of inositol phosphates from the cells. For example, in a previous study, we demonstrated that platelet activating factor-induced phos-

phoinositide metabolism in Kupffer cell occurs in two phases (Gandhi et al., 1990). Extracellular Ca^{2+}-independent formation of inositol bis- and trisphosphates, which occurs within seconds of platelet activating factor challenge is EGTA-insensitive, and is inhibited by an intracellular Ca^{2+} channel antagonist 3,5,5-trimethoxybenzoic acid-8(diethylamino)-octyl ester. On the other hand, formation of glycerophosphoinositol and inositol mono- and bisphosphates, which occurs during stimulation of Kupffer cells for several min with platelet activating factor, is inhibited by EGTA (Gandhi et al., 1990). Although platelet activating factor causes both intracellular Ca^{2+} mobilization and influx of Ca^{2+} from the extracellular medium (Gandhi et al., 1990), stimulation of Kupffer cells with platelet activating factor did not result in the release of inositol phosphates into the medium (Figs. 1 and 4). Therefore, A23187-induced release of inositol phosphates may not be a function simply of elevated $[Ca^{2+}]_i$, but of an additional effect of the ionophore. The precise mechanism by which A23187 causes the release of cellular inositol phosphates is not known. It is very likely that the mechanism may involve permeability to small molecules such as inositol phosphates through membrane pores in A23187-treated cells. These pores are presumably not large enough to permeate large molecules (e.g., proteins) as illustrated by the lack of release of lactate dehydrogenase during the time course of stimulation with A23187.

In summary, our data demonstrate that at the concentrations and the time of incubation tested, A23187 stimulates formation and release of inositol phosphates from the cells without causing an overt cellular injury. Although it appears that A23187 exerts differential effects on phosphoinositide metabolism in various cell types, our work suggests that the interpretation of cellular signaling data generated using cells stimulated with the ionophore should be interpreted following the determination of inositol phosphates in both cells and the culture medium.

Acknowledgements

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