Involvement of external calcium in the release of arachidonic acid by mouse peritoneal macrophages

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The present investigation was undertaken to study the potential role of extracellular calcium on the release of arachidonic acid from mouse peritoneal macrophages. Both in phorbol ester-treated and in Ca^{2+}-depleted cells, a rapid release of arachidonic acid was seen in direct response to added Ca^{2+}. The response was directly dependent on the extracellular Ca^{2+}concentration, with a Ca^{2+} threshold of 100 nM. These results support the notion that arachidonic acid release in macrophages is functionally coupled to influx of external calcium.

Arachidonic acid; Calcium influx; Phospholipase A_2; Mouse macrophage

1. INTRODUCTION

In common with many other cell types, stimulation of phagocytic cells with a wide variety of agonists results in the mobilization of large quantities of AA, as a result of phospholipase A_2 activation [1–4],

Although the regulatory mechanisms which underlie the AA release process are not well understood, a central role for Ca^{2+} in this process is evidenced by the fact that phospholipase A_2 enzymes are strictly dependent on Ca^{2+} [5,6] and their activity is strongly enhanced by cell treatment with calcium ionophores [7]. Since the intracellular Ca^{2+} levels appear to be controlled in part by agonist-induced phospholipase C activation, it has been often thought that phospholipase A_2 activation resulted from prior polyphosphoinositide breakdown by phospholipase C. However, recent findings have questioned this proposal, suggesting that both phospholipases are regulated under separate mechanisms under physiologic stimulation [8].

Further evidence against a causal relationship between phospholipases A_2 and C have come from studies on the effect of phorbol ester treatments on cultured cells prior to a receptor-mediated stimulation [9–12]. In this context, we have recently reported that after a PMA treatment, agonist-induced AA mobilization in mouse macrophages was noticeably enhanced; whereas, agonist-induced inositol phosphates production was greatly inhibited [9]. Thus, PMA treatment dissociates phospholipase A_2 from phospholipase C, showing conclusively that both enzyme activities are under separate but interacting mechanisms of activation [9].

It is known that the rise in the intracellular Ca^{2+} concentration owing to ligand-receptor interaction results from both discharge of internal stores and influx across the plasma membrane. Extracellular calcium seems to be involved in the release of AA in various cell types, including phagocytes [2,9,13,14]. However, the component(s) of the transduction system leading to AA release which is directly affected by external Ca^{2+} has not been clearly identified. In many systems, receptor occupancy is dependent upon the extracellular Ca^{2+} concentration [15], and implications of external Ca^{2+} in the adequate coupling of regulatory components of the transduction cascade have been suggested [16]. On these grounds, the possibility arises that the effect of external Ca^{2+} on receptor-mediated AA release might occur at the level of signaling component(s) and not directly on the amplifying enzyme phospholipase A_2, as has been proposed [9,14]. In order to explore these possibilities, different experimental approaches have been carried out in this study to gain insight into the effect of extracellular Ca^{2+} on AA release in mouse macrophages.

2. MATERIALS AND METHODS

Peritoneal macrophages from Swiss female mice were harvested and purified as described [3,17]. Cell monolayers were incubated at 37°C overnight in a humidified atmosphere at 5% CO_2 and 95% air, in RPMI 1640 medium (Flow Laboratories, Irvine, Scotland, UK), supplemented with 10% (v/v) heat-inactivated fetal calf serum (Flow), penicillin (100 units/ml), streptomycin (100 μg/ml), 2 mM L-glutamine and 0.3 μCi/ml of [^{3}H]AA (New England Nuclear, Boston, MA, USA; sp. act. 76.0 Ci/mmol). At the end of the 16-h

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Abbreviations: AA, arachidonic acid; PMA, phorbol 12-myristate, 13-acetate

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incubation period, macrophages were washed and placed in phosphate-buffered saline solution, containing 1 mM EGTA, 1 mM $MgCl_2$ and variable amounts of $CaCl_2$ for 15 min before addition of 100 ng/ml of PMA (Sigma, St. Louis, MO, USA). After 10 min treatment with the phorbol ester, cells were incubated in the same buffer described above in the presence or absence of 10 μM calcium ionophore, A23187 (Sigma).

To deplete intracellular Ca^{2+}, 2 μM A23187 was added and macrophages were incubated for 15 min at 37°C in the presence of 2 mM EGTA. Cell monolayers were washed 4 times to remove residual Ca^{2+}, placed in a Ca^{2+}-containing medium and then challenged with 10 μM A23187 for 15 min.

For measurements of [^{3}H]AA release, the supernatants were poured off, cleared of cells by centrifugation, mixed with a toluene/Triton X-100 scintillation mixture [18] and assayed for radioactivity.

The Ca^{2+}/EGTA buffers prepared to obtain the desired free Ca^{2+} concentrations, were made according to Raaflaub [19]. Protein was determined according to Bradford [20].

3. RESULTS AND DISCUSSION

Macrophages were treated with PMA (100 ng/ml) during 10 min in order to inhibit polyphosphoinositide breakdown and intracellular Ca^{2+} mobilization [9] and then challenged with the calcium ionophore, A23187 (10 μM), in the presence of different extracellular Ca^{2+} concentrations. As shown in Fig. 1, A23187-induced AA release resulted to be directly dependent on external Ca^{2+} with a threshold concentration of 10^{-7} M (pCa 7). We and others have reported that phospholipase A_2 provides the major route for the release of AA in macrophages, irrespective of the triggering agent used. This is supported by the accumulation of lysophospholipids [2,3,21] and glycerophosphobases [2,21] upon cell activation without apparent losses of AA from monacylglycerol [3,21] or variations in the activity of acyltransferases using AA as donor [22]. Thus, the measurement of [^{3}H]AA release from stimulated macrophages is a useful index of phospholipase A_2. Inasmuch as calcium ionophore, A23187, exerts its stimulatory effects bypassing receptor interaction, the results shown in Fig. 1 are very suggestive of a direct effect of Ca^{2+} on phospholiphase A_2. It is worth noting that the shape of the Ca^{2+} dose-response curve shown in Fig. 1 is very similar to that reported previously for zymosan-stimulated AA release in PMA-treated macrophages, although the ligand provoked a left-shift in the pCa curve [9]. Substantial release of AA was observed in the absence of external Ca^{2+} or when extracellular Ca^{2+} levels were below 10^{-7} M (Fig. 1). This external Ca^{2+}-independent AA release might be due to ionophore-induced disruption of internal Ca^{2+} stores or, most likely, to the potentiating effect of PMA on AA release, as has been described previously [9].

In order to assess directly the role of external Ca^{2+} in AA release while avoiding the external Ca^{2+}-independent mobilization of the fatty acid commented above, mouse macrophages were depleted of their intracellular Ca^{2+} store and then made selectively permeable to external Ca^{2+} by addition of A23187

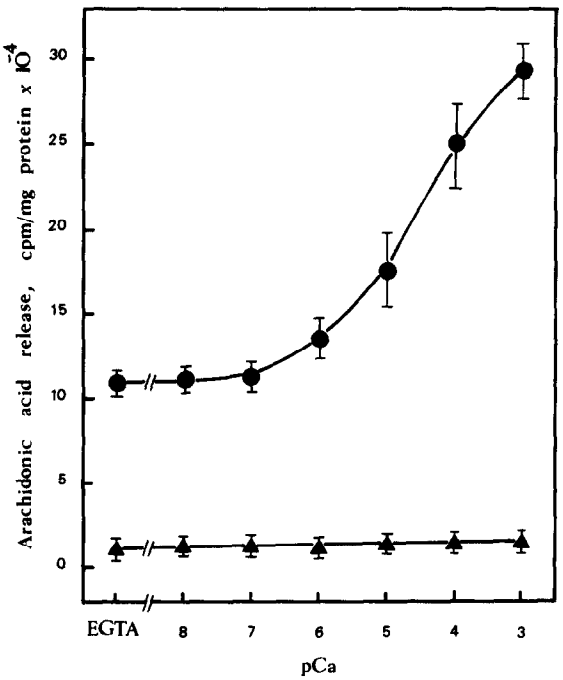

Fig. 1. Effect of extracellular Ca^{2+} concentration on A23187-induced arachidonic acid release. PMA-treated macrophages were incubated with the indicated free Ca^{2+} concentrations in the presence (●) or in the absence (▲) of 10 μM A23187. Incubations were terminated after 15 min and arachidonic acid release into the medium was measured as described in section 2. Results are shown as means ± SE of 3 different experiments.

[16,23,24]. As shown in Fig. 2, AA release occurred when extracellular Ca^{2+} concentration was higher then 10^{-7} M (pCa 7). The response was rapid, displaying nearly the same profile as observed when PMA-treated,

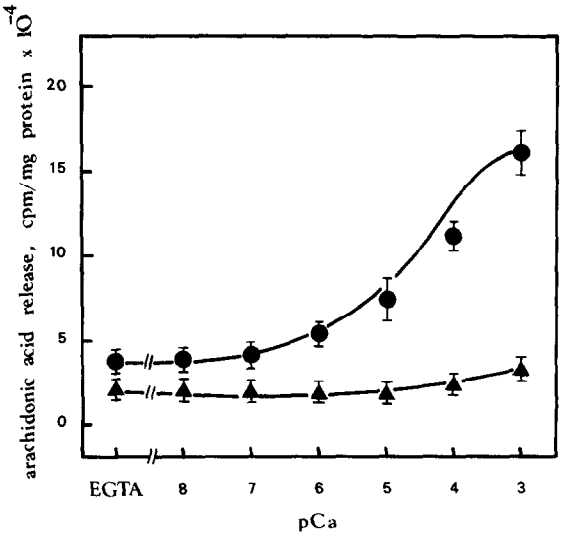

Fig. 2. Arachidonic acid release in Ca^{2+}-depleted macrophages. Ca^{2+}-depleted cells were incubated with the indicated free Ca^{2+} concentrations in the presence (●) or in the absence (▲) of 10 μM A23187. Incubations were terminated after 15 min and arachidonic acid release was measured as described in section 2. Results are shown as mean values ± SE of 3 different experiments.

non-Ca^{2+}-depleted cells were used (cf. Figs 1 and 2). AA released in the presence of millimolar levels of external Ca^{2+} was very close to that observed when non-Ca^{2+}-depleted macrophages (PMA untreated) were stimulated with A23187 (193 000 ± 16 500 cpm/mg protein).

These results demonstrate that a direct correlation exists between stimulated AA release and extracellular Ca^{2+} concentration. Macrophage phospholipase A_2 is thought to constitute the major pathway for AA release [1–6] and this enzyme has been found to require Ca^{2+} for activity [5–7]. Considering that discharge of internal Ca^{2+} stores has been demonstrated not be involved in AA release [9–14], the results herein reported strongly suggest that macrophages maintain their ability to generate AA-derived second messengers by means of the direct and sustained activation of phospholipase A_2 by extracellular Ca^{2+} influx. In support of this view, it is very interesting to note that AA release in this work displays essentially the same calcium dependency as that reported for a partially-purified preparation of macrophage phospholipase A_2 [6]. Moreover, the similarity of the Ca^{2+} dose-response curves for A23187 reported in this work and for zymosan (a receptor-directed stimulus [9], indicates that effects of external calcium at the level of transduction components of the mechanism leading to AA release are not decisive in order to express the cellular AA mobilization response.

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