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PHOSPHOLIPASE C-DIGLYCERIDE LIPASE IS A MAJOR PATHWAY FOR ARACHIDONIC ACID RELEASE IN MACROPHAGES

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Macrophages are a rich source of arachidonic acid oxygenated metabolites and play a remarkable role in a number of physiopathological situations. The synthesis and secretion of arachidonic acid metabolites are triggered at the cytoplasmic membrane level. The present study was outlined to further investigate the cellular mechanisms controlling arachidonic acid release in macrophages. The results presented here strongly suggest that the amount of arachidonic acid released in macrophages in response to phagocytic challenge could be accounted for by a phospholipase C- diglyceride lipase system being unnecesary the presence of phospholipase A_2 whose activity, on the other hand, was found vanishingly small in macrophage homogenates.

Macrophages are a rich source of arachidonic acid active metabolites of both cyclooxygenase and lipoxygenase pathways (1). The synthesis and secretion of these metabolites are triggered, in the phagocytic process, at the plasma membrane level (2). The phosphatidylinositol cycle is an ubiquitous system that has been proposed to control signal transduction through two second messengers (3): diglyceride and Ins 1,4,5-P_3. The former, is an activator of the Ca^{2+} and phospholipid-dependent protein kinase (PKC) (4), and it has also been proposed to be the substrate for arachidonic acid release through a diglyceride lipase system in, for example, platelets (5).

In macrophages, the release of inositol phosphates has recently been shown to occur in response both to pagocytic and ionophoretic stimuli (6,7,8). Therefore, a phospholipase C-diglyceride lipase system might be suggested to account for the release of arachidonic acid in macrophages. However, Wightman et al.(9) were unable to detect diglyceride lipase activity in macrophage homogenates raising the possibility of the non-

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existence of a diglyceride lipase-mediated meaningful pathway for the arachidonic acid release in macrophages. The results presented here reveal that arachidonic acid release in macrophages could be accounted for by a phospholipase C-diglyceride lipase system.

MATERIALS AND METHODS

Macrophage isolation and stimulation

Peritoneal macrophages from Swiss male mice were harvested and purified by adherence to 35 mm plastic culture dishes as described (10). Cells were incubated at 37º C overnight ($4x10^6$ cells/ml) in RPMI 1640 medium (Flow Lab, UK), supplemented with 10 % heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 ug/ml), 20 mM HEPES buffer pH 7.4 and 2 mM L-glutamine. During this incubation period, radioactive precursors were added according to the different experiments described. At the end of the 16 h labeling period, macrophages were washed four times and placed in serum-free medium for 30-60 min before stimulation with zymosan (1 mg/ml) according to the experiments.

Measurement of diacylglycerol and intracellular free arachidonic acid

Diacylglycerol and intracellular free arachidonic acid metabolism was evaluated in macrophages labeled overnight with 5 uCi/ml of (5,6,8,9,11,12,14,15-^{3}H)arachidonic acid. Incubations were stopped and lipids extracted by the method of Bligh and Dyer (11). Lipids were fractionated by thin-layer chromatography by using the following solvent system: hexane/diethylether/acetic acid (60:40:1; v/v/v); after which they were visualized by iodine vapours, scrapped and assayed for radioactivity.

Enzyme activities in macrophage homogenates

Adherence-purified macrophages were scraped with a rubber policeman in Ca^{2+}- and Mg^{2+}-free, EGTA (1 mM)-containing phosphate-buffered saline and centrifuged at 10000 x g for 15 min. Afterwards, macrophages were resuspended in the corresponding assay buffer and sonicated (3 x 30 second pulses) while suspended in a ice bath. Macrophage homogenate protein content was estimated by the Bradford's method by using a commercially available kit (BioRad).

For the measurement of phospholipase A_2, phospholipase C and diglyceride lipase activities an experimental protocol identical to that described elsewhere (12) was followed.

RESULTS

Zymosan (1mg/ml) was found to induce a fast release of radioactivity into the medium in (^{3}H)arachidonic acid-labeled macrophages. This is concomitant to the increase in the radioactivity levels of intracellular free arachidonic acid in the first 30 seconds after the stimulation and the following sharp decrease

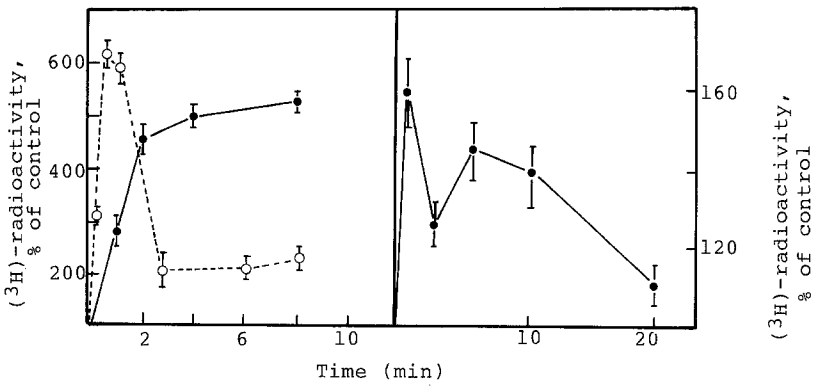

Figure 1. Zymosan-stimulated arachidonic acid release (left) and diglyceride formation (right). Macrophages were labeled overnight with (^{3}H)arachidonic acid, washed and equilibrated as described. Zymosan (1 mg/ml) was added and supernatants were taken at different times and radioactivity content determined by liquid scintillation (●,left). Cellular lipids were extracted and the radioactivity levels of both diglyceride (right) and intracellular free fatty acid (○,left) were determined. Radioactivity content of supernatants, diglyceride and intracellular free fatty acid, in unstimulated controls taken at different times were 23000 ± 300, 2430 ± 230 and 2150 ± 120 dpm/ml, respectively. Results are mean ± SD of three different experiments with incubations in duplicate.

probably as consequence of its metabolization and secretion in the form of prostaglandins and leukotrienes (Fig 1a). Zymosan also induces significant changes in the radioactivity content of diacylglycerol in (^{3}H)arachidonic acid-labeled macrophages (Fig 1b). These data together with the results showing that zymosan activates the release of inositol phosphates in macrophages (6,7,8), strongly suggest that arachidonic release in macrophages could be a phospholipase C-mediated phenomena, as it has been proposed in platelets (5). The use of phospholipase inhibitors to elucidate the contribution of the different arachidonic acid releasing systems has proven to give misleading results in culture cells (12). Then, the only reliable way to investigate the involvement of these enzymatic systems on arachidonic acid release was to determine their activity directly in macrophage homogenates. After establishing linearity assay conditions, V_{max} and K_m for phospholipase C and diglyceride lipase were determined in mouse peritoneal macrophage homogenates. Table 1 clearly shows that while no appreciable phospholipase A_2 activity was detected, phospholipase C and diglyceride lipase activities are high enough as to account for macrophage arachidonic acid release by a phospholipase C-

TABLE 1. Enzyme activities in mouse peritoneal macrophage homogenates

	Vmax*	Km**
Phospholipase C	37.3 ± 0.2	125 ± 9
Diglyceride*** lipase	4.6 ± 0.1	56 ± 3
Phospholipase A_2	ND	

* pmol of product/ug cell protein/min; ** uM; *** free (1-^{14}C)-arachidonic acid is the product determined; ND undetectable; Results are mean ± SD of five independent experiments with incubations in triplicate.

diglyceride lipase system as it is discussed below. It is noteworthy that no an appreciable phospholipase A_2 activity was detected in macrophage homogenates either at pH 4.5 nor at pH 8.5. Moreover, phospholipase A_2 activity was not observed either in a wide range of Ca^{2+} concentrations or in the presence of diglyceride (20 molar %)-a well known inducer of phospholipase A_2 activity (13). Garcia-Gil & Siraganian (14), have recently shown the agonist-activated phospholipase A_2 activity in rat basophilic leukemia cells. In order to test the posibility of zymosan-induced phospholipase A_2 in macrophages, these cells were incubated in the presence of this agonist for different times (0-60 min), following an experimental protocol similar to that described by those authors, after which they were scraped and phospholipase A_2 activity determined as previously described. Under these conditions no activity was found. Furthermore, phospholipase A_2 activity was determined in macrophage homogenates in the presence of zymosan (1 mg/ml) either with or without guanosin-5'-O-(3-thiotriphosphate)(10 uM) in a wide range of Ca^{2+} concentrations (from 100 nM to 10 mM) and, again, no appreciable phospholipase A_2 activity was found. However, it was observed the presence of a small amount of radioactivity in the fatty acid spot in TLC what suggested the presence of phospholipase A_1 activity. This was confirmed by using 1-stearoyl-2(^{3}H)arachidonoyl phosphatidylcholine as substrate. Therefore, it appears clear that in mouse peritoneal macrophages no phospholipase A_2 activity was detected and a low phospholipase A_1 activity was present (Vmax 0.17 pmol/ug protein/min). This activity was not activatable by zymosan in the experiments

described above but it did show a slight Ca^{2+} dependence. When 1-stearoyl-2(^{3}H)arachidonoyl phosphatidylcholine was used as substrate no radioactivity was released into the free fatty acid fraction what strongly suggested the absence of both phospholipase A_2 and lysophospholipase significant activities.

It is worth noting that when phospholipase A_2 from cobra venom was incubated with (^{14}C)diglyceride in the assay buffer for diglyceride lipase, no activity was detected. This clearly support the specificity of the diglyceride lipase assay used in this study. Furthermore, when the cobra venom phospholipase was incubated with either (1-^{3}H)palmitoyl phosphatidylcholine or (2-^{3}H)arachidonoyl phosphatidylcholine, a large degradation of both phospholipids was observed and this experiment was used to assess the actual positional distribution of the radiolabeled fatty into the phospholipid molecule. It is noteworthy that when diglyceride lipase was assayed with 2-(^{14}C)arachidonoyl diglyceride, radiolabeled monoglyceride was found,suggesting the presence of a diglyceride lipase removing the fatty acid from the first position. In order to confirm this, the assay was carried out with 1-(^{3}H)palmitoyl diglyceride. Under these conditions radioactivity was detected only in the free fatty acid fraction. This strongly suggest the existence of a diglyceride lipase activity that removes the fatty acid from the first position but not from the second. This produces 2-arachidonoyl monoglyceride that is the substrate for a monoglyceride lipase that in turn would control the release of arachidonic acid. Therefore the diglyceride lipase activity data given here as (^{14}C)arachidonic acid are actually the resultant of diglyceride lipase-monoglyceride lipase(Table 1).

DISCUSSION

The zymosan-induced changes in the radioactivity content of diglyceride and IP_s confirm the zymosan-stimulated phosphodiesterase attack on phosphoinositides. Previous studies in macrophages suggested a dominating deacylation mechanism to explain the effects of zymosan on the phosphoinositide hydrolysis (6). However, we have been unable to detected either lysophosphatidylcholine, or lysophosphatidylethanolamine or lysophosphatidylinositol in zymosan-activated (^{3}H)glycerol-labeled macrophages (data not shown). The more recent suggestions that a phospholipase A could account for the agonist-stimulated

arachidonic acid release in macrophages (15,16), were, however, drawn from indirect observations (i.e., the diminution of phospholipid radioactivity levels is an average measurement of a complex system that does not rule out the possibility of lipid remodelling that could explain that decrease). Furthermore, the published increases in deacylation products (lysophospholipids, glycerophospholipids) are quite small (near the experimental error range), and therefore their significance is very difficult to ascertain (15,16). It is worth remarking, that our conclusions have been obtained from direct measurements. Our data show that no phospholipase A_2 activity was detected in any condition tested. Wightman et al.(9), has previously reported on the presence of two phospholipase A_2 activities in macrophage homogenates. The reasons for that discrepancy are not clear. However, it is worth remarking here that all the experiments depicted in this paper were carried out under strictly controlled sterility conditions in order to avoid the possible presence of phospholipase A_2 activities from contaminating organisms that would induce to misleading results. When macrophage homogenates were added to an assay with snake venom phospholipase A_2 no inhibition of this activity was observed ruling out the possible presence of an endogenous phospholipase A_2 inhibitor, as it has been proposed to exist in platelets (17,18). Taken together all these results strongly prompt us to think on the actual absence of any significant phospholipase A_2-mediated pathway for arachidonic acid release in macrophages.

A phospholipase C-diglyceride lipase system has been suggested to mediate the release of arachidonic acid in, for example, platelets (5,19). Our data of phospholipase C and diglyceride lipase activities in macrophage homogenates, reveal that the release of arachidonic acid metabolites by these cells in response to a phagocytic stimulus (aproximately 0.4 pmol/ug cell protein/min; see refs. 3 and 20) could be accounted for by a phospholipase C-coupled diglyceride lipase activity. The phospholipase C activity described here is similar to that described by Wightman et al. (21), but while these autors failed to detect any diglyceride lipase activity in mouse peritoneal macrophage homogenates we found activity enough as to account for the arachidonic acid release. It is interesting to note here that reduced gluthatione was added in our assay; the absence of that compound nearly abolished the diglyceride lipase activity.

This could explain the failure of these authors to detect such an activity. It is also noteworthy that the highest of the phospholipase A_2 activities described by Wightman et al. (9) was well bellow our diglyceride lipase activity (0.88 pmol/min/ug protein for phospholipase A_2 vs. 4.6+0.1 pmol/min/ug protein for diglyceride lipase; see ref 12 and Table 1) and according to its characteristics, was clearly of a lysosomal origin.

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