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INTRACELLULAR Ca^{2+} REQUIREMENTS FOR ZYMOSAN-STIMULATED PHOSPHOINOSITIDE HYDROLYSIS IN MOUSE PERITONEAL MACROPHAGES

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The phosphatidylinositol cycle has been demonstrated to be involved in the control of Ca^{2+} cytosolic levels in several cellular types. The Ca^{2+} requirements of phospholipase C activity and the described stimulation of phosphoinositide hydrolysis by Ca^{2+} ionophores make unclear the relationship between phosphatidylinositol cycle and Ca^{2+} mobilization. The results reported here suggest that intracellular Ca^{2+} is necessary for zymosan-stimulated phospholipase C activation in macrophages.
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The phosphatidylinositol cycle has been recognized as an ubiquitous transducting mechanism involved in the regulation of several key cell functions (1,2).

Recent studies have established the phosphodiesteratic attack to phosphatidylinositol-4,5-biphosphate as the foremost event in the chain of processes leading to the cycle activation which yield diglyceride and inositol triphosphate (IP_3) (3). IP_3 would act as a second messenger regulating intracellular Ca^{2+} levels through the stimulation of its release from non-mitochondrial stores (4). On the other hand, it has been suggested the existence of a still unidentified link with Ca^{2+} influx from the extracellular medium (5,6). The Ca^{2+} dependence of the receptor-stimulated phospholipase C activation in several cell systems (7,8) and the fact that Ca^{2+} ionophores seems to stimulate the phosphatidylinositol cycle in several types of cells (9-12), make unclear the relationship between the metabolism of these phospholipids and Ca^{2+} mobilizing mechanisms. Macrophages are immunolo-

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gical cells that play a key role in the regulation of several immunoregulatory processes (13). The phosphatidylinositol cycle has been shown to be activated in these cells by both phagocytic and ionophoretic stimuli (14). The purpose of this study is to investigate the Ca^{2+} requirements of the phospholipase C activity in mouse peritoneal macrophages stimulated both with zymosan and the Ca^{2+} ionophore A23187.

MATERIALS AND METHODS

Peritoneal macrophages from Swiss male mice were harvested and purified as described (15). Cells were incubated at 37ºC overnight in RPMI 1640 (Flow Lab, UK), supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml), 20 mM HEPES pH 7.4, 2 mM L-glutamine and 10 µCi/ml of myo-(2-^{3}H)inositol (Amersham, UK; Sp.Act. 50 Ci/mmol). At the end of the 16-h incubation period, macrophages were washed and equilibrated with serum-free medium for 30-60 min before addition of the different stimulants. Incubations were stopped as described (16) and water-soluble inositol phosphates extracted and fractionated according to (17).

RESULTS AND DISCUSSION

Figure 1 shows that A23187 (10 µM) significantly stimulates the release of water-soluble inositols (IP_s) from (^{3}H)inositol-labeled macrophages during the time of period studied, being the increase of inositol biphosphate (IP_2) observed earlier than that of any other IP_s. This could be interpreted either as a foremost phosphodiesterase attack to phosphatidylinositol-4-phosphate or as a primary degradation of phospatidylinositol-4,5-biphosphate to yield IP_3 which would be quickly degraded, being the system thus deactivated (18).

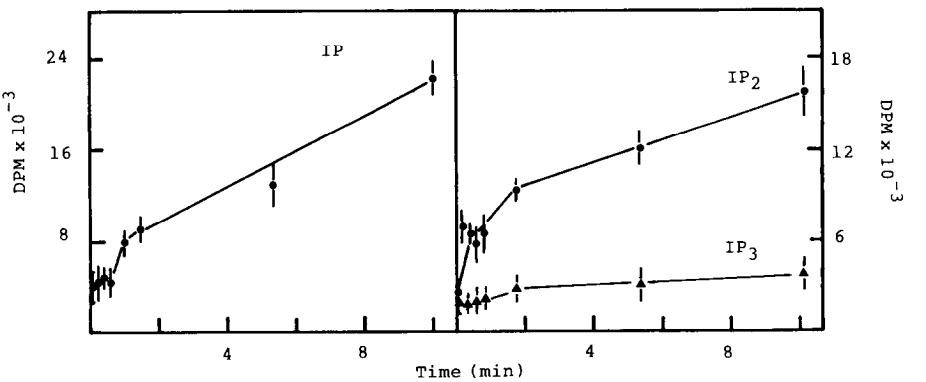

Figure 1. Time course of the effect of A23187 (10 µM) on the accumulation of IP_s. Incubations were carried out in the presence of LiCl (10 mM). The figure shows a typical experiment which was repeated four times with incubations in duplicate.

TABLE 1. Effect of Ca^{2+} and inhibitors of arachidonic acid metabolism on A23187 and zymosan-stimulated IP_s release**

	Incubations in the presence of extracellular Ca^{2+}		Incubations in the absence of extracellular Ca^{2+}	
	None	IND+NDGA*	With intracellular Ca^{2+}	Intracellular Ca^{2+} depleted
None	2500 ± 140	2450 ± 260	1580 ± 120	1480 ± 200
Zymosan (1 mg/ml)	4400 ± 260(76%)+	ND	2550 ± 150(63%)+	1350 ± 260
A23187 (10 μM)	15700 ± 890	16300 ± 460	1600 ± 220	ND

Macrophages were labeled as described and incubated in the presence of extracellular Ca^{2+} or in Ca^{2+}-free, EGTA-containing medium. In depletion experiments, cells were also incubated with A23187 (2 μM). After stimulation (20 min), total IP_s were extracted and fractionated by anion-exchange chromatography.
**Expressed as dpm/well.
*IND, indomethacin (20 μM); NDGA, nordihydroguaiaretic acid (20 μM).
+Percentage of stimulation as related to basal.

Table 1 clearly shows that ionophore-induced phospholipase C activation (as measured by IP_s production) is independent of the production of arachidonic acid metabolites. Taking into account that the concentrations of indomethacin and nordihydroguaiaretic acid used in these experiments completely abolish the metabolism of the liberated arachidonic acid (data not shown), stimulation of labeled macrophages with A23187 in the presence of extracellular Ca^{2+} and indomethacin (20 μM) plus nordihydroguaiaretic acid (20 μM) yields IP_s radioactivity levels similar to those found in the incubations performed in the absence of those inhibitors. Thus, in macrophages, A23187 stimulation of phospholipase C is not secondary to the production of arachidonic acid active metabolites as it has been suggested to occur in platelets (19,20). The absence of Ca^{2+} from the extracellular medium completely suppress the ionophore-induced IP_s release (Table 1). Thus, A23187 does not activate phospholipase C by mechanisms other than Ca^{2+} mobilization. In order to support further the role of Ca^{2+} as activator of IP_s liberation, macrophages were incubated for 10 min in a Ca^{2+}-free, EGTA-containing medium in the presence of A23187 (2 μM), after which Ca^{2+} (1 mM, final concentration) was added and the IP_s production determined. IP_s radioactivity levels under these conditions were very close (14300 ± 650) to those found when A23187 was added in the presence of extracellular Ca^{2+} (Table 1).

On the other hand, incubation of labeled macrophages in a Ca^{2+}-free, EGTA-containing medium slightly decreases but not abolish the zymosan-stimulated IP_s release (63% versus 76% of stimulation over basal values, Table 1). This could be interpreted as a non-absolute Ca^{2+} requirement for zymosan activation of phospholipase C (i.e.,extracellular Ca^{2+} could be necessary for the complete binding of zymosan to its receptor or for the adequate coupling of the components of the transducting system). However, the possibility remains for the requirement of the presence and/or redistribution of intracellular Ca^{2+} to express the IP_s release in zymosan-activated macrophages.

In order to test this last possibility in intact macrophages, intracellular Ca^{2+} stores were depleted by incubating the cells in a Ca^{2+}-free, EGTA-containing medium in the presence of A23187 (2 μM) for 10 min, according to the depletion protocol previously described (21). After this time, cells were stimulated with zymosan (1mg/ml) and the total IP_s release was measured. Under these conditions the macrophage response is completely abolished, suggesting that zymosan-activated IP_s release require the presence and/or mobilization of intracellular Ca^{2+}. However, in other cell types, such as PC12 (22) or T leukaemia (21), this requirement has not been found indicating the different behaviour of phospholipase C in different types of cells.

The fact that the lowest phospholipase C activity observed in our study, belongs to that of Ca^{2+}-depleted cell preparations, point out to this enzyme as the Ca^{2+}-requiring step in the chain of events triggered by zymosan, although the Ca^{2+} dependence of another component of the transducting cascade cannot be completely ruled out.

Finally the results presented in this paper are in good agreement with recently published data (23), which demonstrated that Ca^{2+} is required for phospholipase C activation by the peptide fMet-Leu-Phe in polymorphonuclear leukocyte membrane preparations and that the presence of high Ca^{2+} concentrations (1 mM) activated the enzyme even in the absence of peptide stimulus. Furthermore, our data are also in keeping with those of Irvine et al.(24), who demonstrated that phospholipase C is able to attack its substrate at low Ca^{2+} concentrations when it is a component of a non-bilayer structure but high concentrations of Ca^{2+} can activate the enzyme even though the substrate belongs to a membrane bilayer structure. Something similar to this could be

taking place in our macrophage experimental model. Thus, when cells are stimulated with A23187 the rise in intracellular Ca^{2+} levels would be high enough to activate phospholipase C; but zymosan, acting through a specific receptor-coupling system, would activate IP_s release probably perturbing the membrane bilayer sufficiently to make the substrate available to the enzyme. However, when Ca^{2+} intracellular levels are lowered in depletion experiments, no activity is observed; it means that this ion even at low concentration is required for the zymosan-induced phospholipase C activity. Therefore, phospholipase C seems to be a Ca^{2+}-dependent bu not a Ca^{2+}-activated enzyme by physiological stimulants. However, it cannot be ruled out a previous zymosan-induced Ca^{2+} mobilization from intracellular stores which in turn would activate phospholipase C. Nevertheless, the well known properties of IP_3 as a Ca^{2+} mobilizing agent make unlikely this last possibility (4).

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