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Zymosan-induced release of inositol phosphates at resting cytosolic Ca^{2+} concentrations in macrophages

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Hydrolysis of polyphosphoinositides by phosphodiesterase has been demonstrated to be involved in the control of cytosolic Ca^{2+} concentrations. The stimulation by Ca^{2+} ionophores of the release of inositol phosphates in macrophages, and other cells, together with the Ca^{2+} requirements for zymosan-induced phospholipase C activation, make unclear the relationship between Ca^{2+} mobilization and polyphosphoinositide hydrolysis. The results in the present paper strongly suggest that, for zymosan-induced phospholipase C activation, a previous increase in cytosolic Ca^{2+} is not a required event. These results also show that zymosan-activated release of inositol phosphates may be mediated by a guanine-nucleotide-binding protein.

INTRODUCTION

Phosphoinositide hydrolysis in macrophages has been demonstrated to be activated both by ionophoretic and phagocytic stimuli (Emilsson & Sundler, 1984; Moscat *et al.* 1986*a*). The phosphodiesteratic attack on the PtdIns*P*s produces Ins*P*s and diacylglycerol (Berridge, 1983). One of the former, Ins(1,4,5)P_3, has extensively been demonstrated to regulate cytosolic Ca^{2+} concentrations (Berridge & Irvine, 1984). Diacylglycerol, on the other hand, also plays important cellular functions, both as an activator of protein kinase C (Nishizuka, 1984) and as a source of arachidonic acid in platelets (Bell *et al.*, 1979) and macrophages (Moscat *et al.*, 1986*b*).

Concerning the Ca^{2+} requirements for phosphoinositide hydrolysis, the results so far published appear controversial. Thus the existence of reports supporting the Ca^{2+}-activated release of Ins*P*s, in some cell types, could invalidate, according to some authors (Cockcroft, 1984), the theory whereby phosphoinositide breakdown is the main mechanism controlling the cytosolic Ca^{2+} concentration. Our experience shows that [^{3}H]inositol-labelled macrophages release large amounts of [^{3}H]Ins*P*s in response to A23187 by a mechanism entirely dependent on Ca^{2+} (Moscat *et al.*, 1986*a*). Furthermore, no agonist-induced release of Ins*P*s is observed in Ca^{2+}-depleted macrophages, contrary to what has been observed in PC 12 cells (Vicentini *et al.*, 1985), T leukaemia cells (Sasaki & Hasegawa-Sasaki, 1985), and platelets (Moscat *et al.*, 1986*c*).

The present study was therefore carried out to investigate further the Ca^{2+} requirements for phospholipase C activation in macrophages. Our data suggest that zymosan induces the release of Ins*P*s at resting cytoplasmic Ca^{2+} concentrations to an extent similar to that produced by high Ca^{2+} concentrations. The results presented here suggest that a previous increase in cytosolic Ca^{2+} is not a necessary event for the zymosan-induced breakdown of PtdInsP_2.

MATERIALS AND METHODS

Macrophage isolation and stimulation

Alveolar macrophages from Large White pigs were purified by adherence to 35 mm plastic culture dishes as described by Cohn & Benson (1965). Cells were incubated at 37 °C overnight (4×10^6 cells/ml) in RPMI 1640 medium (Flow Laboratories), supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 units/ml)/streptomycin (100 μg/ml), 20 mM-Hepes buffer, pH 7.4, and 2 mM-L-glutamine. During this incubation period, 10 μCi of [2-^{3}H]inositol (Amersham International; sp. radioactivity 50 Ci/mmol)/ml was added, according to the experiments. At the end of the 16 h labelling period, macrophages were washed four times and placed in a Ca^{2+}-free EGTA (1 mM)-containing phosphate-buffered saline (0.5 mM-$MgCl_2$/0.3 mM-KCl/137 mM-NaCl/8 mM-Na_2HPO_4/1.5 mM-KH_2PO_4, pH 7.4) for 15 min before the addition of A23187 (5 μM). After 10 min treatment with the ionophore, cells were incubated in a cytosol-like buffer (5 mM-$MgCl_2$/120 mM-KCl/20 mM-Hepes, pH 7.4) with different free Ca^{2+} concentrations, either in the presence or in the absence of zymosan (1 mg/ml). After 15 min, reactions were stopped and the Ins*P*s released were determined.

The Ca^{2+} concentration was adjusted by using Ca^{2+}/EGTA buffers and taking into account pH, ionic composition of the media and the association constant of EGTA, as decribed by Raaflaub (1956).

Extraction and fractionation of Ins*P*s

Water-soluble Ins*P*s were extracted and fractionated as described by Berridge *et al.* (1983). The elution pattern of Ins*P*s was previously assessed by using radioactive standards obtained from ^{32}P-labelled erythrocyte ghosts (Downes & Michell, 1981). [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4,5)P_4 were generously provided by Dr. R. F. Irvine.

Abbreviations used: Ins*P*, inositol phosphate; PtdIns, phosphatidylinositol; PtdIns*P*, phosphatidylinositol 4-phosphate; PtdInsP_2, phosphatidylinositol 4,5-bisphosphate; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; GTP[S], guanosine 5'-[γ-thio]triphosphate.

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Fractionation of 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 *g* for 15 min. Afterwards, macrophages were resuspended and sonicated (three 30 s pulses) while suspended in an ice bath. Macrophage homogenates were centrifuged at 100000 *g* for 60 min, and cytosolic and high-speed particulate fractions were isolated. The protein content was measured by Bradford's method, with a commercially available reagent (Bio-Rad).

Phospholipase C assays

Phospholipase C was routinely assayed either with L-3-phosphatidyl[2-^{3}H]inositol (Amersham International; sp. radioactivity 10–15 Ci/mmol) or phosphatidyl [1,2-^{3}H]inositol 4,5-bisphosphate (New England Nuclear; sp. radioactivity 3.6 Ci/mmol) in an assay buffer similar to that described by Irvine *et al.* (1984), also containing 80 mM-KCl/1 mM-$MgCl_2$/50 mM-Tris/maleate, pH 7.4, and substrate (100 μM) either pure or mixed with a 10-fold excess of a PtdEtn/PtdCho mixture of various proportions. In the experiments in which the Ca^{2+}-dependence was assessed, free Ca^{2+} concentrations were obtained as described above. The assays were carried out under the previously established conditions for linearity: 30 μg of cell protein/ml and 10 min. In some experiments, GTP[S] (10 or 100 μM) or sodium taurocholate (5 mM) was added. Zymosan (1 mg/ml) was used as the agonist in these assays. In the experiments in which the hydrolysis of endogenous Ptd[^{3}H]Ins*P*s was assayed, a [^{3}H]inositol-labelled high-speed particulate fraction was isolated from labelled macrophages; reactions were performed in the presence or in the absence of GTP[S] (10 μM), either with or without zymosan (1 mg/ml), for 15 min. Reactions were stopped as described by Watson *et al.* (1984); aqueous phases were used for the determination of Ins*P*s, whereas the chloroform phases were dried down under N_2 and PtdIns*P*s were analysed. Unlabelled PtdIns and PtdInsP_2 were purified from an ox brain Folch fraction by chromatography on immobilized neomycin as previously described (Schacht, 1978).

Fractionation of PtdIns*P*s

The dried lipid extracts were spotted on silica-gel G t.l.c. plates (Merck) pre-sprayed with 1% potassium oxalate. They were developed in chloroform/methanol/water/NH_3 (sp.gr. 0.880) (45:35:8:2, by vol.). Lipids were detected by exposing the plates to iodine vapour, after which they were scraped and their radioactivity content was determined by liquid-scintillation spectrometry.

RESULTS

[^{3}H]Inositol-labelled macrophages were treated with the Ca^{2+} ionophore A23187 (5 μM) for 10 min in accordance with a previously described protocol (Moscat *et al.*, 1986*a*,*c*). Afterwards, different Ca^{2+} concentrations were added by using Ca^{2+}/EGTA buffers, either in the presence or in the absence of zymosan (1 mg/ml), and total Ins*P*s released were determined. Fig. 1 shows that very high Ca^{2+} concentrations produced an extensive liberation of Ins*P*s, in keeping with our previously published data (Moscat *et al.*, 1986*a*). Zymosan is unable

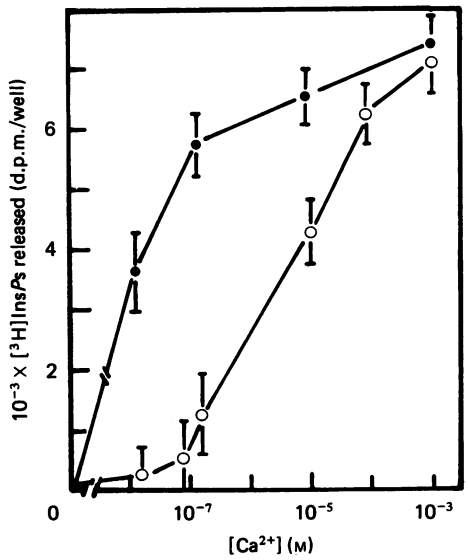

Fig. 1. Effect of Ca^{2+} and zymosan on release of Ins*P*s in A23187-treated macrophages

[^{3}H]Inositol-labelled macrophages were treated with A23187 (5 μM) for 10 min in a Ca^{2+}-free EGTA (1 mM)-containing buffer, after which different free Ca^{2+} concentrations were added, by using Ca^{2+}/EGTA buffers, in the presence (●) or in the absence (○) of zymosan (1 mg/ml). Total [^{3}H]Ins*P*s released were determined as described in the Materials and methods section. Each point is the mean ± S.D. for three independent experiments, with incubations in duplicate.

to produce the release of Ins*P*s in the presence of a Ca^{2+}-free EGTA-containing buffer (Fig. 1). However, at resting cytosolic Ca^{2+} concentrations (80 nM), an appreciable stimulation by zymosan is observed. Therefore, it appears that zymosan is able to induce phosphoinositide hydrolysis at resting cytosolic Ca^{2+} concentrations to an extent similar to that produced by high Ca^{2+} concentrations.

In order to investigate in more detail the Ca^{2+} requirements for the breakdown of PtsInsP_2 and PtdIns by phospholipase C, the following series of experiments was performed. Phospholipase C activity was determined for these two substrates in macrophage homogenates over a wide range of Ca^{2+} concentrations. Fig. 2 shows that the phosphodiesteratic breakdown of PtdIns is strongly Ca^{2+}-dependent, whereas that of PtdInsP_2 is only slightly dependent on Ca^{2+}. It is also noteworthy that the curve shown in Fig. 2 for PtdIns is biphasic.

Some authors have suggested the existence of two types of phospholipase C, i.e. cytosolic and membrane-bound (Friedel *et al.*, 1969; Michell & Lapetina, 1972; Cockcroft *et al.*, 1984). In order to investigate this possibility in macrophages, cell homogenates were centrifuged, and both a cytosolic and a high-speed particulate fraction were isolated. Fig. 3 shows the Ca^{2+}-dependence of the cytosolic enzyme with both substrates. Again, the phosphodiesteratic degradation of PtdIns is dependent on Ca^{2+}, although a clear inhibition is observed at very high Ca^{2+} concentrations (1 mM). It is noteworthy that no such inhibition is observed when the assay was performed with total homogenate (Fig. 2). It could be argued that this lack of inhibition at

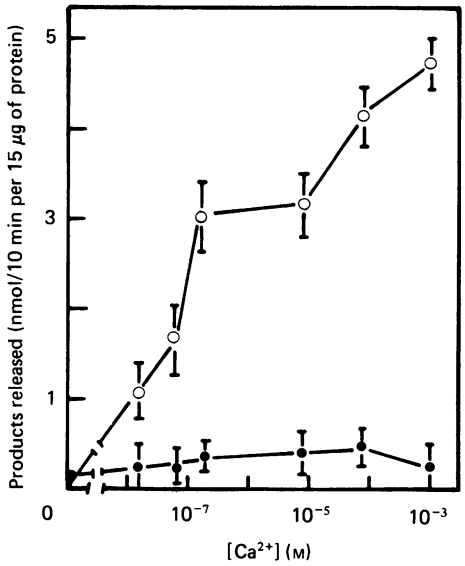

Fig. 2. Effect of different Ca^{2+} concentrations on the phospholipase C activity of macrophage homogenates

Macrophage homogenates (30 μg of cell protein/ml) were incubated for 10 min with either PtdIns (100 μM) (○) or PtdInsP_2 (100 μM) (●). Each point is the mean ± S.D. for three independent experiments, with incubations in duplicate.

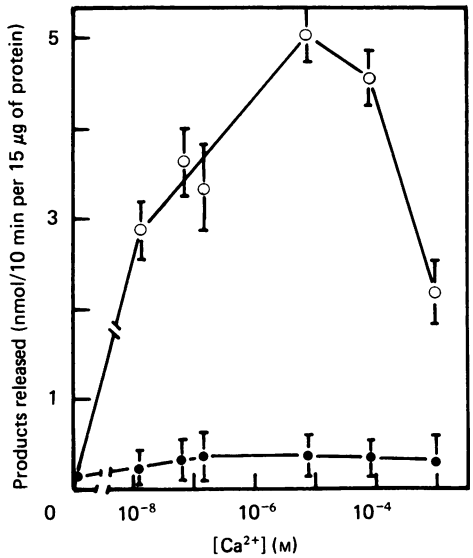

Fig. 3. Effect of Ca^{2+} on the phospholipase C of macrophage cytosol

Macrophage cytosol (30 μg of protein/ml) was incubated for 10 min with either PtdIns (100 μM) (○) or PtdInsP_2 (100 μM) (●). Each point is the mean ± S.D. for three independent experiments, with incubations in duplicate.

1 mM-Ca^{2+} in total homogenates could be due to the presence, in membranes, of a phospholipase C activity that would be activatable only by very high Ca^{2+} concentrations. This possibility is easily ruled out, since, when phospholipase C was assayed with either PtdIns or PtdInsP_2 in the high-speed particulate fraction, no activity was detected towards PtdIns, and only PtdInsP_2 was broken down (0.16 ± 0.01 nmol of product/10 min per 15 μg of cell protein), at any Ca^{2+} concentration tested. It is noteworthy that the specific activity with PtdInsP_2 in the membrane fraction is very similar to that found in total homogenates (0.20 ± 0.03 nmol of product/10 min per 15 μg of cell protein). This strongly suggests that the activity detected, by using exogenous substrates, in the particulate fraction may be merely entrapped cytosolic enzyme. Alternatively, the existence is possible, in macrophage membranes, of some phospholipase C activity that could only be detected after agonist stimulus. In this regard, the receptor-mediated activation of phospholipase C attack on exogenous PtdIns$P$s in blowfly salivary-gland membranes has been reported (Litosch & Fain, 1985). To test this possibility in macrophages, either total homogenates or membrane particulate fractions were incubated at different Ca^{2+} concentrations in the presence or in the absence of GTP[S] (100 μM) and either in the presence or in the absence of zymosan (1 mg/ml). No activation of the enzyme was observed with either PtdIns or PtdInsP_2 in any condition. Furthermore, the presence of taurocholate (5 mg/ml) activated neither the enzyme nor its stimulation by zymosan. However, it is possible that, for the receptor-mediated phospholipase C activation in macrophage membranes, it would be necessary that the substrates belong to the membrane structure and that this structural situation could not be mimicked by the action of detergents. To test this possibility, macrophages were labelled overnight with [^{3}H]inositol, after which they were washed, scraped and homogenized, and a [^{3}H]inositol-labelled high-speed membrane fraction was prepared. Table 1 shows that high Ca^{2+} concentrations activate the release of [^{3}H]Ins$P$s from endogenous Ptd[^{3}H]Ins$P$s in macrophage membranes. Interestingly, zymosan induces release of Ins$P$s (in the presence of 10 μM-GTP[S]), at cytosolic Ca^{2+} concentrations to an extent similar to that produced by very high Ca^{2+} concentrations (1 mM; see Table 1). Furthermore, at zero Ca^{2+} concentration, no zymosan-stimulated liberation of Ins$P$s is observed. It is noteworthy that zymosan in the absence of GTP[S] is unable to activate phosphoinositide hydrolysis. Table 2 shows the changes in endogenous Ptd[^{3}H]Ins$P$s under the same experimental conditions as those shown in Table 1. A significant ($P < 0.001$)

Table 1. [^{3}H]Ins$P$s release in response to different Ca^{2+} concentrations and agonist stimulus in [^{3}H]inositol-labelled macrophage membranes

[^{3}H]Inositol-labelled membranes were isolated from labelled macrophages as described in the Materials and methods section. Each assay contained 30 μg of membrane protein (8000 d.p.m.). Results are expressed as d.p.m./assay, and are means ± S.D. for three independent experiments, with incubations in duplicate.

Addition	[Ca^{2+}] ...	Release (d.p.m./assay) 0	80 nM	1 mM
None		550 ± 60	600 ± 30	1600 ± 148
GTP[S] (10 μM)		580 ± 45	660 ± 46	1600 ± 98
Zymosan (1 mg/ml)		560 ± 30	640 ± 50	1580 ± 80
Zymosan + GTP[S]		560 ± 50	1450 ± 86	1660 ± 110

Table 2. Amounts of Ptd[^{3}H]Ins*P*s in response to Ca^{2+} and agonist stimulus in [^{3}H]inositol-labelled macrophage membranes

[^{3}H]Inositol-labelled membranes were isolated as described in the Materials and methods section. Each assay contained 30 μg of membrane protein (8000 d.p.m.). Results are expressed as d.p.m./assay, and are means ± S.D. for three independent experiments, with incubations in duplicate. Key: N, macrophage membranes; R, macrophage membranes + GTPγ[S] (10 μM) + zymosan (1 mg/ml).

	Content (d.p.m./assay)		
$[Ca^{2+}]$ . . .	0	80 nM	1 mM
PtdInsP_2	N 437 ± 30	478 ± 65	450 ± 38
	R 440 ± 40	210 ± 23	280 ± 40
PtdIns$P$	N 427 ± 20	410 ± 34	460 ± 30
	R 430 ± 35	420 ± 30	430 ± 25
PtdIns	N 6780 ± 300	7200 ± 200	5050 ± 200
	R 6600 ± 430	6700 ± 250	4700 ± 210

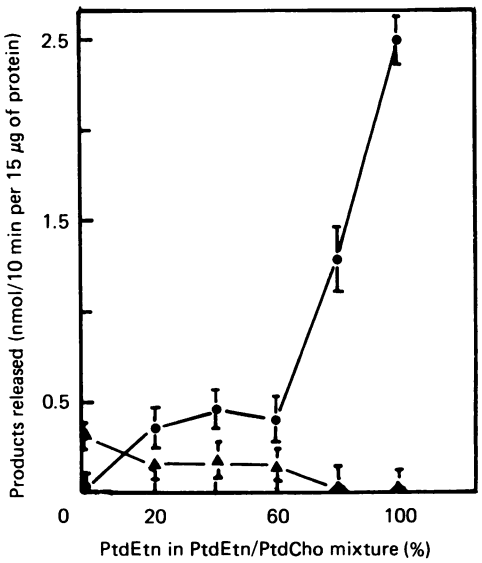

Fig. 4. Effect of PtdCho/PtdEtn mixtures on the macrophage cytosol phospholipase C activity

The substrate was presented mixed with a 10-fold excess of PtdCho/PtdEtn in various proportions, and the incubations were performed as described in the legend of Fig. 2, with 80 nM-Ca^{2+}: ▲, PtdIns; ●, PtdInsP_2. Each point is the mean ± S.D. for three independent experiments, with incubations in duplicate.

decrease was observed in Ptd[^{3}H]Ins when labelled membranes were incubated at 1 mM-Ca^{2+}, this decrease being even larger in the presence of zymosan plus GTP[S]. Interestingly, a significant decrease ($P < 0.005$) was observed in Ptd[^{3}H]InsP_2 when the labelled membranes were incubated with zymosan plus GTP[S] at resting cytosolic Ca^{2+} concentrations.

It has been suggested that the phosphodiesteratic breakdown of PtdInsP_2 could be accomplished by a rat brain cytosolic phospholipase C, at low Ca^{2+} concentrations, when PtdInsP_2 belonged to a destabilized membrane structure (Irvine *et al.*, 1984). This suggests that the ligand promotes the phosphodiesteratic attack on PtdInsP_2 by inducing a previous destabilization of the membrane that would make the enzyme accessible to the substrate. Fig. 4 shows that when PtdIns and PtdInsP_2 belong to a bilayer structure (0–20 mol% PtdEtn in a PtdCho/PtdEtn mixture) a small fraction of substrate is hydrolysed at resting cytosolic Ca^{2+} concentrations. However, when the membrane bilayer is destabilized, by increasing its PtdEtn content, a clear activation of the PtdInsP_2 breakdown is observed, whereas PtdIns degradation is not activated.

DISCUSSION

The results presented in this paper clearly indicate that zymosan, at Ca^{2+} concentrations reported to exist in the cytosol of resting cells (approx. 80 nM), induces the release of Ins*P*s to a similar extent to that produced by very high Ca^{2+} concentrations. This has been shown by using both ionophore-treated [^{3}H]inositol-labelled macrophages and membranes from these labelled cells. Furthermore, at cytosolic Ca^{2+} concentrations, the hydrolysis of PtdInsP_2 is significantly activated by zymosan (Table 2). Similar findings have been described in chemoattractant-activated phospholipase C in polymorphonuclear leucocyte membranes (Smith *et al.*, 1985), although the Ca^{2+} concentration reported by those authors as necessary for the agonist-stimulated breakdown of PtdInsP_2 was well above the cytosolic Ca^{2+} value. Our data (Fig. 4) and those of Irvine *et al.* (1984) show that phospholipase C hydrolyses PtdInsP_2 extensively, at cytosolic Ca^{2+} concentrations, when the substrate belongs to an unstabilized membrane structure. PtdIns, however, does not seem to be regulated by the degree of membrane structure, at least in macrophages, whereas Ca^{2+} concentrations actually appear to control the phospholipase C-catalysed hydrolysis of PtdIns. Several systems have been reported to require guanine nucleotides for the agonist-stimulated activation of phospholipase C (Haslam & Davidson, 1984; Litosch & Fain, 1985; Merritt *et al.*, 1986). In our cellular system, GTP[S] is necessary for zymosan-stimulated hydrolysis of PtdIns*P*s. Taken together, all these data permit one to speculate on the possible mechanism whereby zymosan stimulates phosphoinositide hydrolysis. Thus zymosan, through a guanine-nucleotide-binding protein, and possibly through membrane destabilization, activates the phosphodiesterase attack on PtdInsP_2 without a previous increase in cytosolic Ca^{2+}. The release of InsP_3 would raise the cytosolic Ca^{2+} concentrations, which in turn would activate the phospholipase C-catalysed PtdIns hydrolysis. The diacylglycerol produced through this pathway would be hydrolysed by diacylglycerol lipase, the main pathway controlling arachidonic acid release in macrophages (Moscat *et al.*, 1986*b*). This would be in good agreement with the hypothesis outlined by Majerus *et al.* (1985).

The fact that PtdInsP_2 is hydrolysed at resting cytosolic Ca^{2+} concentrations (in the presence of zymosan plus GTP[S]) and that PtdIns is hydrolysed at high Ca^{2+} concentrations might explain the agonist-induced biphasic production of diacylglycerol in hepatocytes (Bocckino *et al.*, 1985), smooth-muscle cells (Griendling *et al.*, 1986) and macrophages (Moscat *et al.*, 1986*b*).

On the other hand, it is noteworthy that Creutz *et al.* (1985) have reported the activation of the phospholipase C of chromaffin-granule binding proteins through its Ca^{2+}-dependent binding to chromaffin membranes. Our data in Figs. 2 and 3 strongly suggest that the presence of macrophage membranes would positively modulate the phosphodiesteratic breakdown of PtdIns. Whether or not this positive modulation is produced by enzyme translocation is a matter that deserves further research. In this regard, it is noteworthy that phorbol esters activate arachidonic acid release in macrophages (Aderem *et al.*, 1986), and the enzymes regulated by these tumour promoters, such as protein kinase C, are activated by translocation (Wolf *et al.*, 1985).

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