

# Stereoselective effects of lysophosphatidylserine in rodents

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**1** The pharmacological action of the L- and D-enantiomers of lysophosphatidylserine has been studied *in vivo* by following the increase in blood and brain glucose content caused by this phospholipid in mice. Preliminary experiments have confirmed that these effects are the consequence of lysophosphatidylserine-induced mast cell activation since they are not observed in mast cell-deficient mice bearing the W/W<sup>o</sup> genotype.

**2** Maximal hyperglycaemic response and brain glucose accumulation occur at 10 mg kg<sup>-1</sup> lysophosphatidyl-L-serine (i.v.). Half-maximal effect is at 3.5 mg kg<sup>-1</sup>. Lysophosphatidyl-D-serine at doses of up to 25 mg kg<sup>-1</sup> i.v. elicits 40% (blood glucose) and 60% (brain glucose) of the maximal effect. The difference in activity between the two enantiomers is also observed in the desensitization to lysophosphatidylserine occurring when this phospholipid is administered by the oral route.

**3** Lysophosphatidyl-L-serine is more active than the D-enantiomer in mouse isolated peritoneal mast cells. Activity ratios of 10 are observed between 20 and 50% histamine release. Similar results are obtained with rat isolated peritoneal mast cells.

**4** It is concluded that the configuration of the alpha carbon atom of serine influences the activity of lysophosphatidylserine *in vivo* and *in vitro*. Thus, the appropriate position of the serine amino group is required for optimal interaction of the phospholipid head group and a receptor in the mast cell membrane.

## Introduction

Although phosphatidylserine comprises a minor fraction of membrane phospholipids, its regulatory function is central to cell activity. Three areas of influence have been defined. In mammals, phosphatidylserine is an intermediate in the synthesis of a major component of membrane phospholipids, phosphatidylethanolamine (Voelker, 1984). Key membrane-bound enzymes, notably protein kinase C and transport ATPase are optimally activated by this phospholipid (Palatini *et al.*, 1972; Takay *et al.*, 1979). In addition, enhanced phosphatidylserine hydrolysis in damaged cells may promote intercellular communications through the generation of the soluble derivative lysophosphatidylserine. In this last effect, lysophosphatidylserine acts as an autacoid (Bruni *et al.*, 1986). In rodents, the preferential target cell for lysophosphatidylserine is the mast cell. These cells are activated *in vivo* (Bigon *et al.*, 1979; Bruni *et al.*, 1984)

and *in vitro* (Martin & Lagunoff, 1979; Smith *et al.*, 1979). The mechanism of the lysophosphatidylserine-mast cell interaction is not completely clear. The central problem is whether the phospholipid acts after incorporation and subsequent perturbation of membrane properties such as permeability and enzyme activity or as a true autacoid, thus activating specific receptors located on the mast cell surface. A detailed study of the structure-activity relationship *in vitro* supports the possibility that a receptor is involved in the lysophosphatidylserine-induced activation of mast cells (Horigome *et al.*, 1986; Tamori-Natori *et al.*, 1986). Optimal mast cell stimulation is observed when (a) the alpha carbon atom of serine is in L-configuration, and (b) an ester bond links the carbon in position 1 of glycerol (stereo-specific numbering) to a fatty acyl chain of 16–18 carbon atoms. Furthermore, mast cell activation is induced by lysophosphatidylserine analogues that cannot be converted into phosphatidylserine. These findings prompted the present

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study aimed at investigating whether stereoselectivity is also manifest in the *in vivo* action of lysophosphatidylserine.

## Methods

### *In vivo experiments*

Outbred male albino mice of 23–26 g (Charles River, CrI:CD-1 (ICR)BR) or mast cell-deficient mice (W/W<sup>v</sup>, Jackson Lab.) were used. Some tests were also performed in female inbred mice, strain C57BL/6 NCrlBR (Charles River). The lysophosphatidylserine solution was injected into the tail vein ( $100\ \mu\text{l}\ 10\ \text{g}^{-1}$ ) and the mice killed 10 min later for blood histamine determination. The blood from 10 mice was pooled to obtain material sufficient for a duplicate test. Alternatively, mice were decapitated 30 min after the injection to collect the blood and to allow the head to fall in liquid nitrogen. Blood glucose was extracted with 0.33 M perchloric acid. To measure brain glucose the hemispheres were removed from the frozen heads and powdered together with 0.5 ml of frozen 0.66 N perchloric acid. The samples were then allowed to warm to 0°C. The concentration of perchloric acid was reduced to 0.33 N and the samples centrifuged. Glucose was determined in the supernatant. Individual determinations were possible in this test.

### *In vitro experiments*

Purified rat peritoneal mast cells and a mixed population of mouse peritoneal cells were obtained as described previously (Boarato *et al.*, 1984). Rat mast cells ( $2.5 \times 10^5$ ,  $2\ \mu\text{g}$  of histamine base) or mouse peritoneal cells ( $4 \times 10^6$ ,  $0.8\ \mu\text{g}$  of histamine base) were incubated for 15 min at 37°C in 0.5 ml of a saline solution containing (mM): NaCl 140,  $\text{KH}_2\text{PO}_4$  2.7,  $\text{Na}_2\text{HPO}_4$  10, KCl 2.7,  $\text{CaCl}_2$  1, glucose 10 and bovine serum albumin  $1\ \text{mg ml}^{-1}$  (pH 7.2). Albumin was omitted in the experiments with mouse mast cells. After centrifugation, the released histamine was determined in the supernatant. The histamine release has been expressed as a percentage of total mast cell histamine measured by boiling the cell suspension for 10 min in 0.1 M HCl. The unstimulated histamine release was within 10% of total histamine.

### *Analytical procedures*

Blood histamine was purified by the butanol extraction procedure as detailed previously (Bruni *et al.*, 1984). Histamine was then determined by the *o*-phthalaldehyde fluorimetric procedure (Shore *et al.*, 1959). Glucose was measured by a spectrophotometric procedure, following the enzymatic reduction of

NADP (Boehringer, test combination gluco-quant).

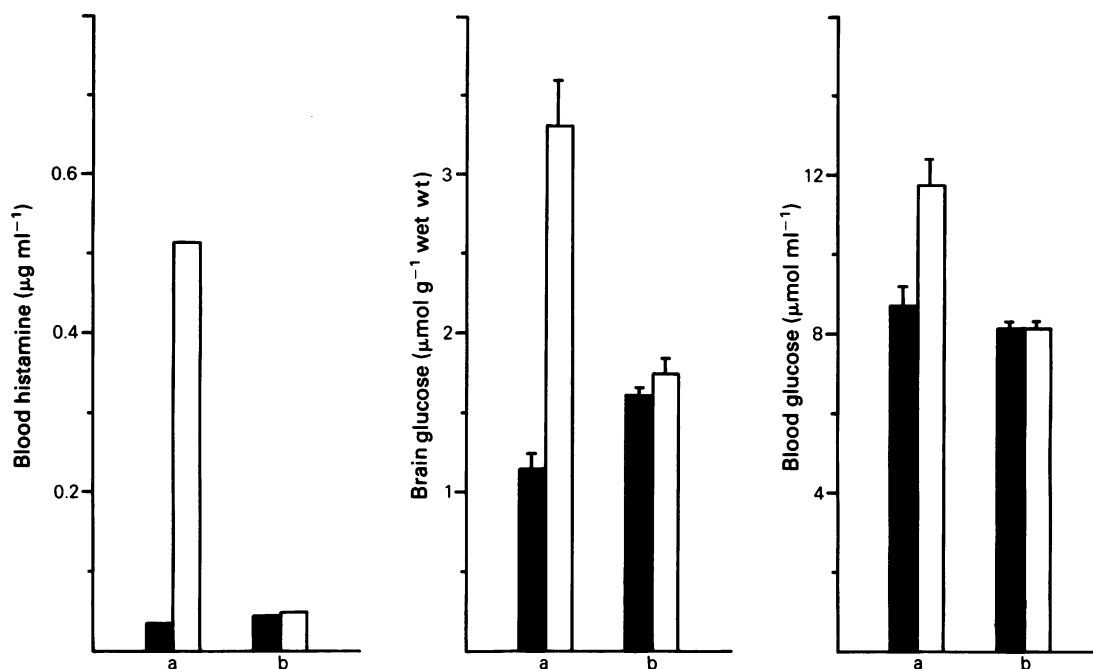
### *Biological compounds*

Phosphatidyl-L-serine and lysophosphatidyl-L-serine were obtained from bovine brain as described (Bigon *et al.*, 1979). Phosphatidyl-D-serine was prepared from egg phosphatidylcholine by the transphosphatidylation reaction catalyzed by phospholipase D in the presence of D-serine, by use of the procedure described by Comfurius & Zwaal (1977). A further supply of phosphatidyl-D-serine was generously provided by Meito Sangyo Co., Tokyo. This phospholipid was converted to lysophosphatidyl-D-serine by the action of phospholipase A<sub>2</sub> from porcine pancreas (Boehringer) and the product was purified by extraction with petroleum ether-diethyl ether (1:1, v/v) (Holub & Piekarski, 1979). The final product, dissolved in chloroform/methanol (2/1, v/v), was washed with 0.2 vol of 0.05 M HCl. Thin layer chromatography showed a single spot. When needed, the desired amount of lysophospholipid was taken to dryness from a solution in chloroform/methanol and dissolved in 50 mM Tris HCl (pH 7.8). The phospholipid solution was used without delay. Nerve growth factor (2.5 S NGF) was prepared from mouse submaxillary glands as described previously (Bruni *et al.*, 1982).

## Results

### *In vivo experiments*

Confirming previous observations (Bigon *et al.*, 1979; Bruni *et al.*, 1984), Figure 1 shows that lysophosphatidyl-L-serine ( $5\ \text{mg kg}^{-1}$ , i.v.) produced a 16 fold increase in the blood histamine level in normal albino mice (CrI:CD-1(ICR)BR). This effect was followed by hyperglycaemia and brain glucose accumulation. Separate experiments showed that the same effects were induced in C57BL/6 NCrlBR mice, an alternative strain of mice normal with respect to mast cell content. By contrast, lysophosphatidylserine did not increase the blood histamine nor induced changes in the blood and brain glucose content in W/W<sup>v</sup> mice. Since determinations of blood and brain glucose offered a simple and reproducible test of lysophosphatidylserine action *in vivo*, the subsequent experiments were performed using this method. As shown in Figure 2, a difference was manifest in the action of L- and D-enantiomers of lysophosphatidylserine. On brain glucose the dose-response curve for lysophosphatidyl-L-serine was bell-shaped. Maximal effect (4.4 times increase) was attained at  $10\ \text{mg kg}^{-1}$ , i.v. Half-maximal effect occurred at  $3.5\ \text{mg kg}^{-1}$ . By contrast, the D-enantiomer induced only 60% of maximal brain glucose increase at  $25\ \text{mg kg}^{-1}$ . Higher doses of this



**Figure 1** Action of lysophosphatidylserine in normal (a) and  $W/W^v$  (b) mice. Histamine was determined in pooled blood of 10 mice, 10 min after the i.v. injection of  $5 \text{ mg kg}^{-1}$  lysophosphatidyl-L-serine. Brain and blood glucose were determined 30 min after the same dose of the phospholipid. Means for 8–15 mice with s.e. mean shown by vertical lines. Solid columns, untreated mice; open columns, mice treated with lysophosphatidylserine.

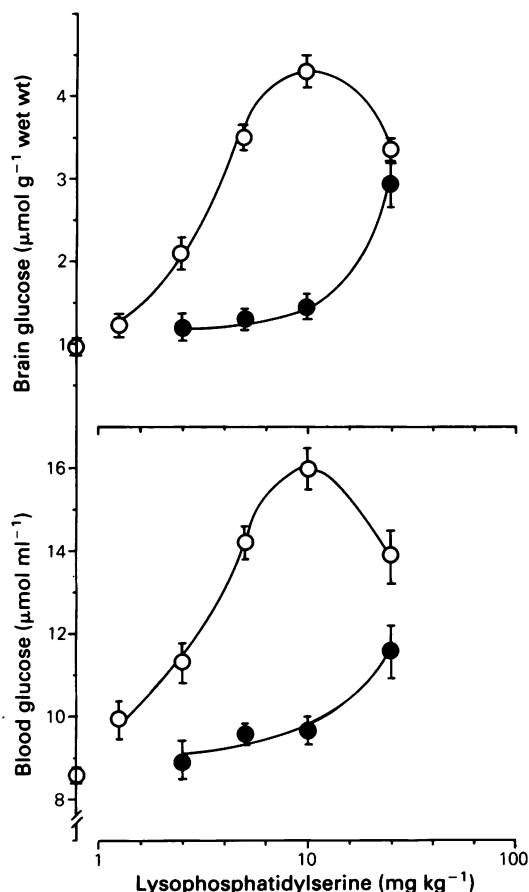
compound could not be tested due to the appearance of a strong haemolytic effect. The increase of blood glucose elicited by lysophosphatidyl-L-serine closely paralleled that produced on brain glucose. An increase of 1.9 times was observed at peak effect. Half-maximal increase was at  $3.5 \text{ mg kg}^{-1}$ . In this test, lysophosphatidyl-D-serine ( $25 \text{ mg kg}^{-1}$ ) elicited only 40% of maximal effect.

Previous experiments (Bruni & Toffano, 1982) showed that lysophosphatidylserine administered by the oral route did not change the blood and brain glucose. However, a dose-dependent desensitization was obtained with intravenous lysophosphatidylserine administration, given between 3 and 18 h later. In accord, lysophosphatidyl-L-serine ( $5 \text{ mg kg}^{-1}$ , i.v.) was less effective on brain glucose if given to mice pretreated orally 16 h beforehand with phospholipid  $25 \text{ mg kg}^{-1}$  (Figure 3). Considering the net increase elicited by lysophosphatidylserine, it can be calculated that the effect of phospholipid was 55% decreased in desensitized animals ( $P < 0.01$ ). Although with lower efficiency, some desensitization was produced also by lysophosphatidyl-D-serine (29% inhibition,  $P < 0.05$ ). Separate controls showed that lysophosphatidyl-L-

serine ( $25 \text{ mg kg}^{-1}$ ) given by the oral route produced a small, though inconstant, effect on blood histamine. A maximal rise of 2.5 times was observed 60 min after its administration.

#### *In vitro experiments*

To investigate the correlation between the activity of lysophosphatidylserine enantiomers *in vivo* and *in vitro* these compounds were also examined in isolated cell preparations. As shown in Figure 4a, lysophosphatidyl-L-serine ( $0.25$ – $4.0 \mu\text{M}$ ) promoted the secretion of 75–80% mast cell histamine in 15 min of incubation at  $37^\circ\text{C}$ . By contrast, up to  $8 \mu\text{M}$  lysophosphatidyl-D-serine elicited only 50% histamine release. Higher concentrations could not be tested since it is known that cytolytic effects are predominant as the concentration of lysophospholipid approaches the critical micellar concentration. In rat mast cells, cytotoxic concentrations of lysophosphatidylserine are around  $10 \mu\text{M}$  (Martin & Lagunoff, 1979). In the range of 20–50% histamine release, activity ratios of 10 were consistently found between the two enantiomers (Figure 4b). Similar results were obtained in

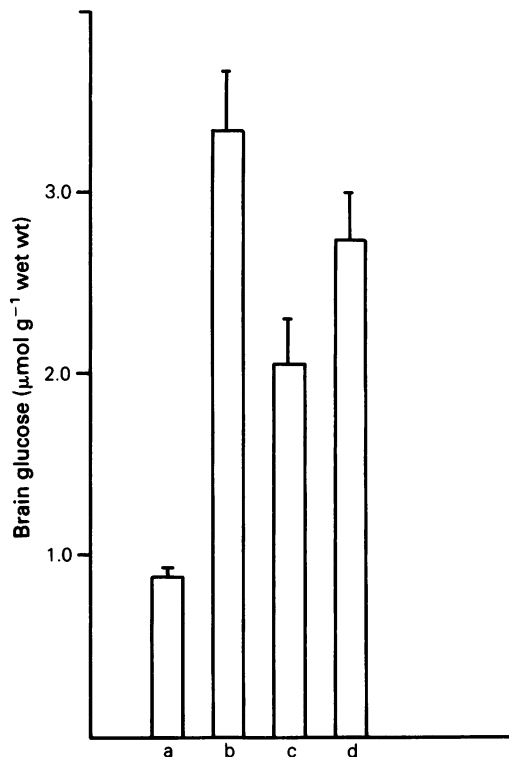


**Figure 2** Action of lysophosphatidyl-L-serine (O) and lysophosphatidyl-D-serine (●) in mice. The indicated dose of the two enantiomers was injected in the tail vein. Blood and brain glucose were measured 30 min later. Means for 15–20 mice; vertical lines show s.e.mean.

purified rat mast cells (Figure 5) when measuring the lysophosphatidylserine- and nerve growth factor-dependent histamine release (Bruni *et al.*, 1982).

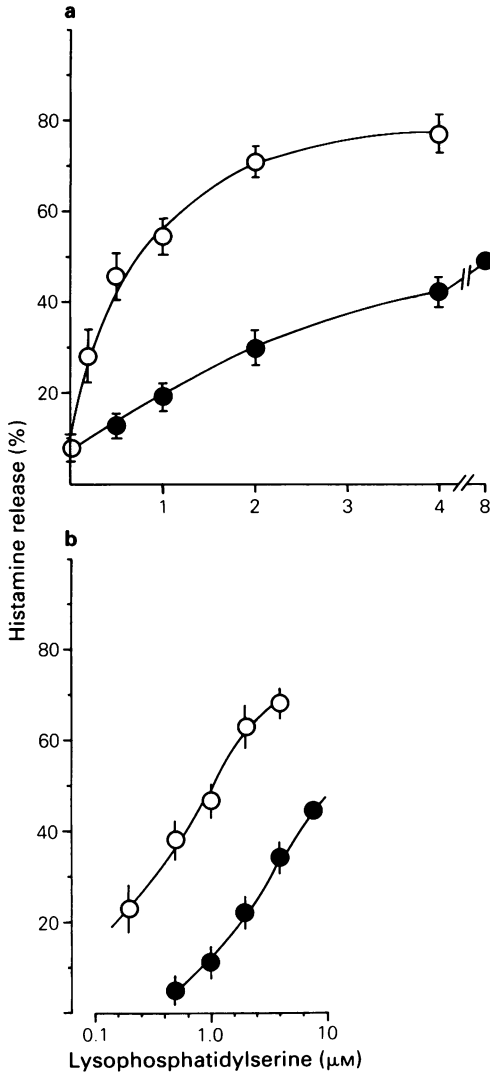
## Discussion

The stereoselectivity in the effects of lysophosphatidylserine *in vivo* allows clarification of basic aspects of the pharmacological action of this phospholipid. Since enantiomers have identical chemical and physical properties but differ only in the spatial arrangement of functional groups, stereoselectivity excludes the possibility that the action of lysophosphatidylserine is due to non specific effects such as influence of negative charges or perturbation of cell



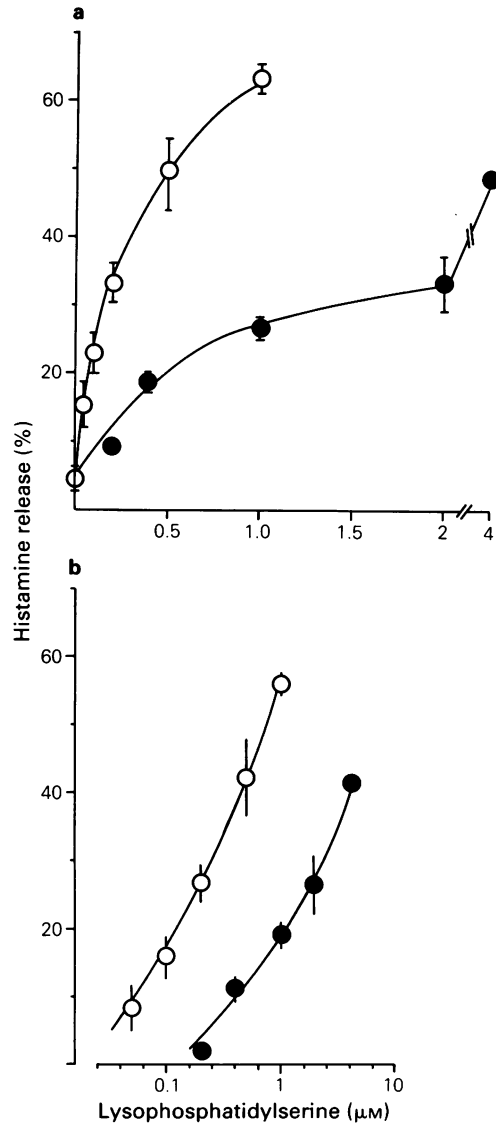
**Figure 3** Desensitization to lysophosphatidylserine induced by oral administration of  $25 \text{ mg kg}^{-1}$  of the two enantiomers to mice. After 16 h,  $5 \text{ mg kg}^{-1}$  of lysophosphatidyl-L-serine were injected i.v. to the same animals. Brain glucose was determined 30 min later. (a) Untreated mice, (b) mice treated only with lysophosphatidyl-L-serine i.v., (c) lysophosphatidyl-L-serine orally, then lysophosphatidyl-L-serine i.v. and (d) lysophosphatidyl-D-serine orally, then lysophosphatidyl-L-serine i.v. Means for 15 mice with vertical lines showing s.e.mean.

membrane structure. This conclusion is in agreement with previous findings showing that the action of lysophosphatidylserine is not reproduced by other lysophospholipids (Bigon *et al.*, 1979). In the present study, the lysophosphatidylserine-induced activation of mast cells *in vivo* has been evaluated by the effect on blood and brain glucose content. As shown previously (Bruni *et al.*, 1984), hyperglycaemia results from the adrenal stimulation induced by circulating mast cell mediators whereas accumulation of glucose in the brain is the consequence of a general depressive state. In accordance with this, the present results show that glucose redistribution is not observed in mast cell-deficient mice bearing the W/W<sup>v</sup> genotype. In these animals the number of skin mast cells is less than 1% of that in normal mice whereas in other tissues these



**Figure 4** Histamine release from mouse mast cells. Mouse peritoneal cells ( $4 \times 10^6$  of which 5% were mast cells) were incubated for 15 min at  $37^\circ\text{C}$  in 0.5 ml of buffered saline solution containing 1 mM  $\text{CaCl}_2$  and 10 mM glucose. (a) Total histamine release; (b) net (elicited minus spontaneous) histamine release. (O) Lysophosphatidyl-L-serine; (●) lysophosphatidyl-D-serine. Means for 4 experiments with vertical lines showing s.e.mean.

cells are absent (Kitamura *et al.*, 1978). In comparison to the L-enantiomer, lysophosphatidyl-D-serine is less potent in changing the blood and brain glucose content although the effect on the brain is more obvious. The different effects in the two compartments



**Figure 5** Histamine release from rat mast cells. Purified rat peritoneal mast cells ( $2.5 \times 10^5$ ) were incubated for 15 min at  $37^\circ\text{C}$  in 0.5 ml of saline solution containing 1 mM  $\text{CaCl}_2$ , 10 mM glucose, 1.5 nM 2.5 S nerve growth factor and  $1 \text{ mg ml}^{-1}$  bovine serum albumin. (a) Total histamine release; (b) net (elicited minus spontaneous) histamine release. (O) Lysophosphatidyl-L-serine; (●) lysophosphatidyl-D-serine. Means for 3 experiments with vertical lines showing s.e.mean.

can be understood if the small size of the free glucose pool in the brain relative to the large size of the glucose pool in the blood is considered. This makes the measurement of brain glucose changes a more sen-

sitive test for the action of lysophosphatidylserine. The highest dose of lysophosphatidyl-D-serine that could be used intravenously ( $25 \text{ mg kg}^{-1}$ ) produces 60% of maximal brain glucose increase and 40% of hyperglycaemic response. Considering equieffective doses (Figure 2), it may be concluded that lysophosphatidyl-D-serine is 6–8 times less active than the L-enantiomer. Stereoselectivity *in vivo* may be the consequence of different isomer pharmacokinetics. We have evaluated this possibility by comparison of our isomer preparations *in vivo* and *in vitro* in isolated mast cells. With mouse and rat peritoneal mast cells, activity ratios of 10 between the two enantiomers are consistently found, confirming the data of Horigome *et al.* (1986). This value is in agreement with the *in vivo* observations and shows that phospholipid disposition is not responsible for the different activity of L- and D-enantiomers. Rather, the data suggest a stereospecific interaction between the phosphoserine head groups and a functional receptor site involved in the events leading to release of mast cell mediators. Since the only difference between lysophosphatidyl-L-serine and lysophosphatidyl-D-serine is in the configuration of the alpha carbon atom of serine, it is likely that the alpha amino group participates in the interaction of the phospholipid with this receptor. This possibility is in agreement with *in vitro* and *in vivo* observations (Martin & Lagunoff, 1979; Bruni *et al.*, 1984) showing loss of activity in N-acetyl or N-acetimidyl lysophosphatidylserine. The lysophosphatidylserine-sensitive site in the mast cell secretory sequence is at present undetermined. Since in rat peritoneal mast cells the phospholipid is not active in the absence of other mast cell ligands such as concanavalin A, dextran and nerve growth factor, lysophosphatidylserine is generally

regarded as a modulator, rather than a promoter of histamine secretion. However, the high activity of this compound in mouse mast cells in the absence of other secretagogues (Boarato *et al.*, 1984) shows that the step influenced by lysophosphatidylserine is in the main pathway of mast cell secretory activity. An interesting property of lysophosphatidylserine action is the desensitization manifest after a single administration of phospholipid. Tachyphylaxis after an intravenous administration of lysophosphatidylserine has been described earlier (Bruni *et al.*, 1984). However, in this case the desensitization follows a phase of systemic manifestations due to massive release of mast cell mediators. The results of this paper show that desensitization also occurs when the phospholipid is given by the oral route, in the absence of overt manifestations of anaphylaxis. Thus, a protracted flow of limited amounts of phospholipid from the intestinal depot into the general circulation is sufficient to induce an appreciable depletion of mast cell mediators. This possibility is indicated by the small rise in the blood histamine level observed in some animal group after the oral administration of lysophosphatidylserine. In addition a first contact with the phospholipid may result in the desensitization of lysophosphatidylserine receptor site. Desensitization in mast cells after a prior exposure to secretagogues has been reported (Foreman & Garland, 1974; Morrison *et al.*, 1975). Since tachyphylaxis is more manifest at high agonist concentrations, rapid desensitization may account for the bell-shaped lysophosphatidylserine dose-response curve.

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