

# Phospholipase A<sub>2</sub> inhibition prevents mucosal damage associated with small intestinal ischaemia in rats

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**SUMMARY** The influence of various inflammatory inhibitors on the damaging effects of ischaemia in the small intestinal mucosa has been investigated. A rat experimental model was used, in which a ligated loop of the distal ileum was subjected to ischaemia and revascularisation and the ensuing mucosal damage assessed by lysosomal enzyme release and intestinal permeability measurements. The mucosal content of malondialdehyde – a lipid peroxidation product – and its activity of myeloperoxidase – a neutrophil granulocyte marker was also determined. In the absence of inhibitor, ischaemia and revascularisation caused increased mucosal permeability to sodium fluorescein, increased N-acetyl-β-glucosaminidase release from the mucosa into the lumen, increased malondialdehyde content in the mucosa and increased myeloperoxidase activity in the mucosa. All these effects were inhibited by the phospholipase A<sub>2</sub> inhibitors, quinacrine and nordihydroguaiaretic acid (NDGA), while the lipoxygenase inhibitor, BW755C, had no influence and the cyclooxygenase inhibitor, indomethacin, potentiated the increases in mucosal permeability and N-acetyl-β-glucosaminidase release. BN 52021, a specific platelet activating factor antagonist, did not influence the myeloperoxidase activity, but it decreased the formation of malondialdehyde and the increases in mucosal permeability and N-acetyl-β-glucosaminidase release, although not to the same extent as quinacrine and NDGA. These findings indicate that phospholipase A<sub>2</sub> inhibition prevents mucosal damage associated with small intestinal ischaemia and suggest that at least part of the ischaemic damage is mediated by products of phospholipase A<sub>2</sub> activity that are not arachidonic acid metabolites.

The mechanisms underlying mucosal injury caused by small intestinal ischaemia have not been elucidated. Oxygen derived free radicals produced in the tissue during a postocclusive hyperaemia have been put forward<sup>1</sup> but the precise molecular mechanisms by which the free radicals might injure the intestinal mucosa have not been substantiated. Recently,<sup>2</sup> we found that the intestinal permeability increase after ischaemia was potentiated by lysophosphatidylcholine, a potentially membrane damaging surfactant occurring naturally in the gut<sup>3</sup> but also known to

induce mucosal injury at higher concentrations.<sup>4</sup> We also found that ischaemia and revascularisation in the small intestine caused not only accumulation of malondialdehyde in the mucosa, but also increased activity of phospholipase A<sub>2</sub>, decreased activity of lysophospholipase, and increased ratio between lysophosphatidylcholine and phosphatidylcholine. Moreover, the intestinal mucosa could be protected against ischaemic injury by quinacrine, a phospholipase A<sub>2</sub> inhibitor.<sup>5</sup> These findings, taken together, suggest that activation of phospholipase A<sub>2</sub> may play a role in ischaemic intestinal injury, a possibility that would be consistent with the recent findings that lysophosphatidylcholine and other lysophospholipids accumulate in the heart after ischaemia

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and are important mediators of sequelae after myocardial ischaemia.<sup>6</sup>

The reason why phospholipase A<sub>2</sub> activation may be important in mediating ischaemic intestinal injury is unclear, however. Phospholipase A<sub>2</sub> is involved in the formation of several inflammatory promoting and potentially toxic agents, such as arachidonic acid metabolites, lysophospholipids, and platelet activating factor. In addition, phospholipase A<sub>2</sub> activation may serve a physiological purpose by eliminating lipid peroxidation products originating in membranes due to free radical propagation.<sup>7-9</sup> Moreover, it is not known whether the phospholipase A<sub>2</sub> activation is confined to intestinal epithelial cells or if other cells, invading the mucosa might contribute to the phospholipase A<sub>2</sub> activation and/or the mucosal damage.

The present investigation was conducted with a view to get more detailed information about the role of phospholipase A<sub>2</sub> activation in the pathogenesis of small intestinal ischaemic damage. To this end, we examined the influence of various inhibitors of lipid metabolism on ischaemic damage to the mucosa, and we also studied the influence of such inhibitors on lipid peroxidation and leucocyte infiltration in the mucosa after ischaemia and revascularisation. Our findings indicate that at least part of the ischaemic damage is caused by products of phospholipase A<sub>2</sub> activity, but that these products are lysophospholipids and/or platelet activating factor rather than arachidonic acid metabolites.

## Methods

### CHEMICALS

The materials and their sources were as follows: sodium fluorescein (E Merck, Darmstadt, FRG); phospholipase A<sub>2</sub> (*Naja naja*) and nordihydroguaiaretic acid (NDGA) (Sigma Chemical Co, St Louis, Mo, USA); quinacrine, and BW755C (Wellcome Laboratories, UK); and indomethacin (Confortid R, Dumex A/S, Denmark). BN 52021 was generously provided by Dr Pierre Braquet of IHB-IPSEN Research Laboratories, Le Plessis Robinson, France.

### ANIMALS AND EXPERIMENTAL DESIGN

A rat experimental model described in detail elsewhere<sup>10</sup> was used. The model is based on tenting the mesenteric vessels to a ligated loop of the ileum and, after a certain time, lowering the vessels down again. Previous studies have shown that this procedure causes ischaemia and revascularisation in the intestinal mucosa, and that the ensuing mucosal damage can be assessed by determining lysosomal enzyme release and increase in mucosal permeability.<sup>10</sup>

In order to study how various agents influenced the ischaemic damage, anaesthetised rats were given 500 µl saline solution containing the agent under study. The following agents were given: quinacrine (10 mg/kg); NDGA (12 mg/kg); BW755C (12 mg/kg); indomethacin (10 mg/kg), and BN 52021 (3.2 mg/kg and 4.8 mg/kg, respectively). Control animals were given saline only. The solution was given intravenously 30 minutes before preparing the ligated loop. Thereafter, to create total ischaemia in the loop, the mesenteric vessels were heightened 2 cm and kept in that position for two hours. The vessels were then brought down again and the gut segment revascularised for five minutes. Control animals were prepared the same way but the mesenteric vessels were left untreated for two hours and five minutes. The extent of mucosal damage was then assessed by determining the activity of N-acetyl-β-glucosaminidase in the gut lumen. In addition, the mucosal contents of malondialdehyde, myeloperoxidase, and in some cases phospholipase A<sub>2</sub> was determined.

For these chemical determinations, the loop was excised and the luminal contents gently removed. After centrifugation of the luminal fluid at 2800 g for 5 minutes, the supernatant was withdrawn and kept at -20°C until analysed. The mucosa was washed with cold saline and scraped off with a curette; special precaution was taken to remove only the superficial layers of the mucosa. The mucosal cells were suspended in 150 mM NaCl, weighed, and disintegrated in a Dounce homogeniser by five strokes with a Teflon pestle. The homogenised cells were then also kept at

Table Influence of various inhibitory agents on luminal N-acetyl-β-glucosaminidase (NAG), mucosal malondialdehyde (MDA) and mucosal myeloperoxidase (MPO) activity in the rat small intestine after ischaemia. The animals were pretreated with intravenous injections of the different agents and the small intestine subjected to ischaemia and revascularisation

Pretreatment	NAG*	MDA†	MPO‡
Saline/ischaemic control	1.9 (0.2)	5.7 (0.6)	141.2 (19.0)
Quinacrine	0.3 (0.1)§	1.3 (0.2)§	20.0 (7.8)§
NDGA	0.4 (0.2)§	1.4 (0.2)§	14.3 (3.5)§
BW 755C	2.1 (0.2) NS	5.3 (0.4) NS	137.3 (19.6) NS
Indomethacin	3.2 (0.2)	5.4 (0.4) NS	133.1 (20.8) NS
BN 52021 (3.2 mg/kg)	0.9 (0.1)§	3.7 (1.0)	Not determined
BN 52021 (4.8 mg/kg)	0.9 (0.1)§	3.6 (0.6)	142.0 (14.4) NS
Saline (non-ischaemic control)	0.2-0.1§	1.3 (0.3)§	11.5 (2.0)§

\*µkat/l; †nmol/mg protein; ‡units/mg protein; §(p<0.001 as compared with the saline/ischaemic control); ||(p<0.01 as compared with the saline/ischaemic control).

Values means (SD) of four-five animals in each group. NS=not significantly different from the ischaemic control.

-20°C until analysed. N-acetyl-β-glucosaminidase (NAG), malondialdehyde (MDA), and myeloperoxidase (MPO) were determined as described elsewhere.<sup>11-13</sup> Protein was determined according to Lowry *et al.*<sup>14</sup>

#### PERMEABILITY MEASUREMENTS

In another set of experiments, the extent of mucosal damage was assessed by determining the passage of permeability marker molecules across the gut wall.<sup>10</sup> Animals were prepared as above and the ileal loop subjected to total ischaemia for 30 minutes. The portal vein was then cannulated with a Venflon cannula (diameter 1.00 mm) and 3.3 mM sodium fluorescein in 1 ml 150 mM NaCl was instilled in the ligated loop. Blood samples (100 μl) were withdrawn from the portal vein before deposition and at 10

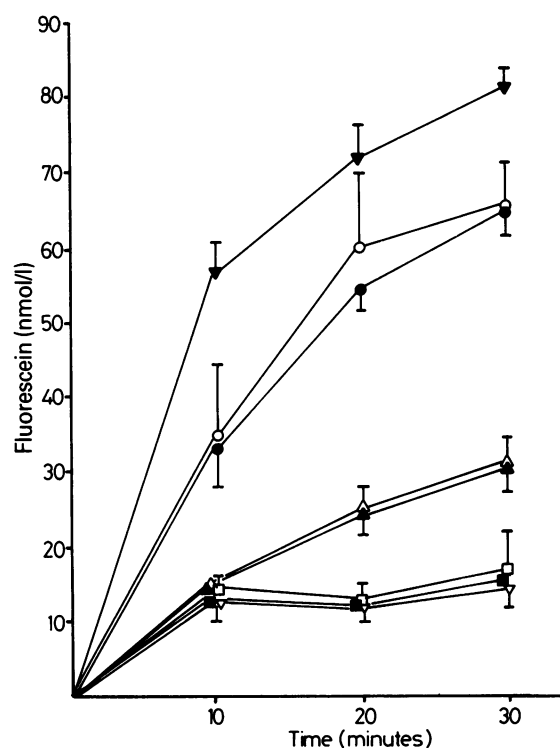


Fig. 1 Influence of various inhibitory agents on small intestinal permeability after ischaemia. Rats were given intravenous injections of the different agents (▼ = indomethacin, ○ = saline, ● = BW755C, △ = BN 52021 (3.2 mg/kg), ▽ = BN 52021 (4.8 mg/kg), □ = NDGA, ■ = quinacrine) and a gut segment subjected to total ischaemia for 30 minutes. The gut segment was then filled with sodium fluorescein and the concentration of fluorescein in the portal blood determined at different times thereafter. Means of 4-5 animals in each group, vertical bars indicate standard deviation. ▽ = non-ischaemic controls given saline.

minute intervals for 30 minutes. The samples were mixed with 1.9 ml Tris (50 mM)-NaCl (150 mM), pH 10.3, centrifuged (2800 g, 5 minutes), and the supernatant analysed for sodium fluorescein using fluorescence spectrometry.<sup>15</sup>

#### DETERMINATION OF PHOSPHOLIPASE A<sub>2</sub> INHIBITION

Experiments were also designed to investigate the influence of NDGA and BW755C on purified (Sigma) phospholipase A<sub>2</sub> activity. NDGA, BW755C and appropriate controls were first incubated (37°C, 10 minutes) with 5 mU phospholipase A<sub>2</sub> in 500 μl 150 mM NaCl containing 100 mM Tris-HCl, pH 7.4, and 5 mM CaCl<sub>2</sub>. To the mixtures were then added 10,000 cpm <sup>14</sup>C-oleate-labelled *E coli*<sup>16</sup> and the mixtures were incubated at 37°C for another 60 minutes. The reactions were then stopped by addition of 3 ml 150 mM NaCl with 1% bovine serum albumin and the phospholipase A<sub>2</sub> activity determined by filtration through a 0.45 μm Millipore filter. The activity was expressed as per cent of the activity in the absence of inhibitor.

Phospholipase A<sub>2</sub> activity was also determined in homogenised mucosal cells from rats given NDGA (12 mg/kg), quinacrine (10 mg/kg), or saline before subsection to small intestinal ischaemia for two hours and revascularisation for five minutes. The reaction mixtures contained, in a final volume of 450 μl, <sup>14</sup>C-oleate-labelled *E coli* (4000 cpm), 70 mM Tris-maleate, pH 7.2, 2 mM CaCl<sub>2</sub>, 0.01% (wt-vol) bovine serum albumin, and mucosal cells corresponding to 4 μg protein. The mixtures were incubated at 37°C and the phospholipase A<sub>2</sub> activity determined as described above. One unit of enzyme activity was defined as liberation of 1% of the total radioactivity into the Millipore filtrate during 90 minutes incubation.

#### STATISTICAL ANALYSIS

Significance of differences were calculated using Student's *t* test.

#### Results

##### PERMEABILITY MEASUREMENTS

The intestinal permeability to sodium fluorescein in the different animals is illustrated in Figure 1. The ischaemia caused a substantially increased permeability in animals pretreated with saline; this increase, however, did not occur in animals given quinacrine or NDGA. On the other hand, BW755C did not influence the permeability increase, and indomethacin rather potentiated it. BN 52021 reduced the permeability increase after ischaemia, although not to the same extent as quinacrine and NDGA.

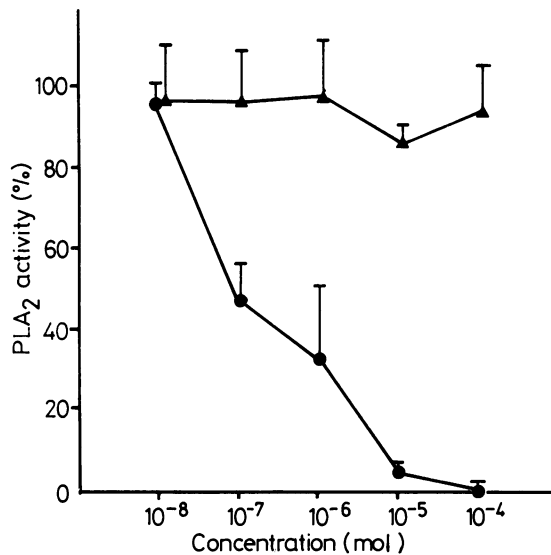


Fig. 2 Influence of BW755C (▲) and NDGA (●) on purified phospholipase A<sub>2</sub> activity. The activity of 5 mU Sigma phospholipase A<sub>2</sub> was taken as 100%. Means of three experiments, vertical bars indicate standard deviation.

#### NAG, MDA AND MPO DETERMINATIONS

The Table shows luminal NAG, mucosal MDA and mucosal MPO activity in the different animals. In animals given saline, the ischaemia caused a profound increase in all three variables. As compared with the values obtained in these ischaemic controls, however, the values in animals pretreated with quinacrine or NDGA were much lower, almost as low as in non-ischaemic controls. By contrast, BW755C-treatment had no influence and indomethacin, which also had no influence on mucosal MDA and MPO, rather increased luminal NAG over ischaemic control values. BN 52021 reduced luminal NAG and mucosal MDA accumulation, although not to the same extent as quinacrine and NDGA. On the other hand, the mucosal MPO activity in animals given BN 52021 before ischaemia was as high as in ischaemic controls.

#### INFLUENCE ON PHOSPHOLIPASE A<sub>2</sub> ACTIVITY

The influence of NDGA and BW755C on purified phospholipase A<sub>2</sub> activity is shown in Figure 2. Thus, NDGA inhibited the activity in a dose dependent fashion whereas BW755C had no effect.

The influence of quinacrine and NDGA on phospholipase A<sub>2</sub> activity in the mucosa after ischaemia is depicted in Figure 3. The ischaemia caused a marked increase in phospholipase A<sub>2</sub> activity in animals pretreated with saline, but not in animals pretreated with quinacrine or NDGA. The mucosal phos-

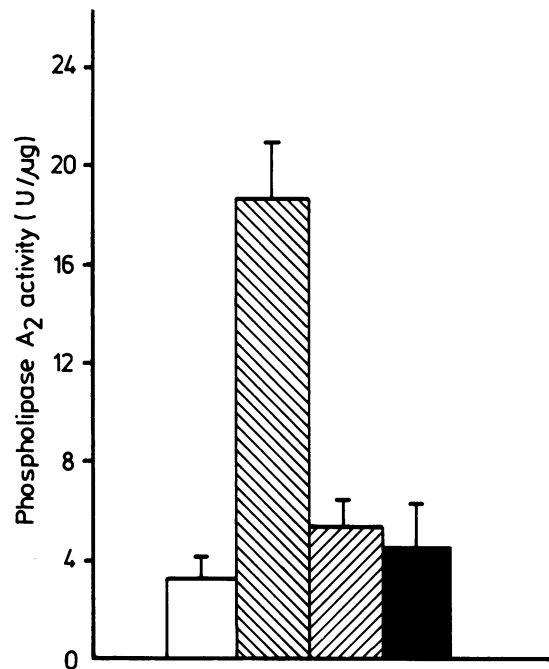


Fig. 3 Influence of quinacrine and NDGA on phospholipase A<sub>2</sub> activity in the intestinal mucosa after ischaemia and revascularisation. Control animals were given saline and the mucosa left untreated (□) or subjected to ischaemia and revascularisation (▨). Experimental animals were given NDGA (▧) or quinacrine (■) before the ischaemia. Mean of five experiments, vertical bars indicate standard deviation.

pholipase A<sub>2</sub> activity remained as low as in non-ischaemic controls in both these groups.

#### Discussion

Recent findings indicate that phospholipase A<sub>2</sub> activation may play an important role in mediating ischaemic intestinal injury.<sup>5</sup> The question arises, therefore, whether inhibition of phospholipase A<sub>2</sub> activation might protect the mucosa against ischaemic damage. In a previous investigation,<sup>5</sup> morphological examinations were used to show that the mucosa could be protected against ischaemic damage by quinacrine, a phospholipase A<sub>2</sub> inhibitor.<sup>5</sup> In the present investigation, we have tried to apply biochemical determinations and permeability measurements to more quantitatively assess the effects of ischaemia and the extent of mucosal damage. We used lysosomal enzyme (NAG) release and intestinal permeability increase as indices of mucosal damage, and MDA level as an indicator of lipid peroxidation. In addition, we determined the mucosal activity of

MPO, a specific marker of polymorphonuclear leucocytes<sup>13</sup>; this was because infiltrating leucocytes have previously been shown to play an important part in ischaemic tissue injury.<sup>17</sup>

The results obtained confirm previous findings in that phospholipase A<sub>2</sub> inhibition prevents mucosal damage associated with small intestinal ischaemia in rats. Quinacrine, which has been shown to inhibit phospholipase A<sub>2</sub> activity in many other systems<sup>18</sup> was thus found to prevent phospholipase A<sub>2</sub> activation and mucosal damage in the present investigation. One possible reason why quinacrine had this protective effect was that it prevented the phospholipase A<sub>2</sub>-dependent release of arachidonic acid and so the formation of inflammatory promoting arachidonic acid metabolites such as leucotrienes. This possibility was first supported by the finding that NDGA, which has been used as a lipoxygenase inhibitor<sup>19</sup> in a number of recent studies, also protected the mucosa. Another lipoxygenase inhibitor, however, BW755C<sup>20</sup> had no such effect (Fig 1 and the Table) and although this could have been due to effects of BW755C on both lipoxygenase and cyclooxygenase<sup>21</sup> that counteracted each other, we suspected that the difference between NDGA and BW755C was because of differences in their phospholipase A<sub>2</sub>-inhibiting capacity. Indeed, this turned out to be the case. Nordihydroguaiaretic, but not BW755C, was thus found to inhibit purified phospholipase A<sub>2</sub> activity, and it also prevented the increase in mucosal phospholipase A<sub>2</sub> activity after ischaemia (Fig. 3). It is suggested, therefore, that phospholipase A<sub>2</sub> inhibition is important for mucosal protection, but probably not because it prevents the formation of leucotrienes. On the other hand, the cyclooxygenase inhibitor, indomethacin, aggravated the mucosal damage, suggesting that prostaglandins may be involved in protecting the mucosa against ischaemic injury.

The experiments did not reveal which specific mechanism(s) caused the phospholipase A<sub>2</sub> activation. One possibility is that reactive oxygen metabolites and free radicals were formed in the mucosa, and that the ensuing peroxidation of fatty acid residues in membrane lipids caused the phospholipase A<sub>2</sub> activation. It has previously been shown that increased phospholipase A<sub>2</sub> activity is detected during the peroxidic decomposition of mitochondrial and erythrocyte membrane lipids, and that the removal of peroxidation products originating in membranes is phospholipase A<sub>2</sub>-dependent. Sevianian *et al* thus showed<sup>7</sup> that phospholipase A<sub>2</sub> may serve to eliminate membrane peroxides, which in turn decompose to yield MDA<sup>22</sup> and Beckman *et al* recently showed that phospholipase A play an integral role in microsomal lipid peroxidation.<sup>9</sup> It is noteworthy, therefore, that the agents confining total mucosal protection and phospholipase A<sub>2</sub> inhibition, quinacrine and NDGA,

also totally prevented MDA formation. This is consistent with previous findings<sup>9,23</sup> and supports the notion that phospholipase A<sub>2</sub> may serve to eliminate lipid peroxidation products originating in membranes due to free radical propagation.

The precise way by which the phospholipase A<sub>2</sub> activation caused mucosal damage was not demonstrated. Increasing numbers of leucocytes, as indicated by MPO activity, were found in the damaged mucosa (Table) and it is possible that they were recruited via a mechanism that involved lipid peroxidation and phospholipase A<sub>2</sub> activation in the mucosal cells. The presence of leucocytes in the damaged mucosa also raises the question whether these cells contributed to the damage or were there because of the damage. It is difficult to distinguish between these possibilities on the basis of the present experiments. The finding that indomethacin-treated and control ischaemic animals showed the same MPO-levels but different degrees of mucosal damage (Table) does not preclude the possibility that leucocytes were important contributors to the damage, because the indomethacin-treatment may well have made the mucosa more vulnerable to the damaging effects of leucocytes. Moreover, the strong correlation between MPO and MDA levels suggests that much of the lipid peroxidation in the mucosa was due to infiltrating leucocytes. Leucocytes are known to harbour efficient systems for free radical generation and secretion<sup>24</sup> and lipid peroxidation yielding MDA could have occurred either inside the leucocytes or in adjacent epithelial cells.

It is also of particular interest that many proinflammatory actions of leucocytes appear to involve phospholipase A<sub>2</sub> activation.<sup>25</sup> Phospholipase A<sub>2</sub> is thus involved in the generation of a variety of inflammatory-promoting and potentially toxic lipids in leucocytes, not only arachidonic acid metabolites but also lysophospholipids and platelet activating factor. We recently found that small intestinal ischaemia and revascularisation caused increased lysophosphatidylcholine:phosphatidylcholine ratio in the mucosa but that this increase did not occur when the mucosa was protected against ischaemic damage by quinacrine.<sup>5</sup> Although it was not shown that these changes related to infiltrating leucocytes, the observations indicated that lysophosphatidylcholine was formed in the mucosa and that it contributed to the damage after ischaemia and revascularisation. The present investigation showed that BN 52021, a specific platelet activating factor antagonist, significantly reduced the permeability increase after ischaemia and that it reduced the increases in N-acetyl- $\beta$ -glucosaminidase release and malondialdehyde accumulation. These findings, therefore, indicate that ischaemia and revascularisation causes the formation of platelet activating factor and that this agent also contributes

to the mucosal damage after ischaemia. The observation that BN 52021 reduced the MDA accumulation without decreasing the MPO activity suggests that one way by which platelet activating factor contributes to the mucosal damage is by stimulating the generation of toxic oxidants in mucosal leucocytes. This would be consistent with the recent observations that leucocytes are primed to release toxic oxidants by contact with thrombin stimulated endothelium and that this is because of endothelial cell generated platelet activating factor.<sup>26</sup>

We have thus obtained some further evidence to indicate that phospholipase A<sub>2</sub> inhibition prevents mucosal damage associated with small intestinal ischaemia and that at least part of the ischaemic damage is mediated by products of phospholipase A<sub>2</sub> activity that are not arachidonic acid metabolites. The reason why phospholipase A<sub>2</sub> inhibition prevents mucosal damage was not revealed, but it is possible that it is because it prevents the free radical induced production of toxic lysophospholipids and/or the leucocyte-dependent generation of platelet activating factor. These possibilities are now being further investigated.

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