Volume 326, number 1,2,3, 222–226 FEBS 12692 July 1993

Phospholipase D treatment enhances gonadotrophin receptor-coupled adenylate cyclase activity in isolated bovine luteal cells

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Received 28 April 1993

LH-stimulated adenylate cyclase activity in membrane preparations of bovine luteal cells could be enhanced by treating the cells with either phospholipase D or its hydrolysis product, phosphatidic acid. Similar augmentary effects were also produced following treatment of the cells with EGF. Moreover, EGF could stimulate the formation of [^{3}H]phosphatidic acid in [^{3}H]myristic acid preloaded cells, suggesting that EGF is able to activate cellular phospholipase D. Also, PMA was able to increase the phosphatidic acid formation with a parallel increase in the adenylate cyclase activity. We propose, therefore, that phosphatidic acid may act as an intracellular second messenger linking EGF-mediated activation of phospholipase D with the sensitization of LH receptor-coupled adenylate cyclase signalling system.

Phosphatidic acid; Cyclic AMP; Phorbol ester; Protein kinase C; Signal transduction

1. INTRODUCTION

Ligands that activate phosphatidyl inositol-specific phospholipase C and protein kinase C often also modulate the adenylate cyclase signalling pathway in the target cells [1–3]. In one report, a direct inhibitory effect of exogenously added phosphatidyl choline-specific phospholipase C from *Bacillus cereus* on adenylate cyclase activity has been demonstrated in 3T3 fibroblasts [4]. In ovarian steroidogenic cells, adenylate cyclase signalling pathway is of primary importance since both the process of differentiation and steroidogenesis are under the control of hormones that elevate cyclic AMP contents of the cells. Thus crosstalks with other signal transduction pathways which result in the modulation of hormone receptor-coupled adenylate cyclase system can be of major consequence in the regulation of various differentiated cell functions. In the case of bovine luteal cells, we have previously demonstrated that pretreatment of the cells with either phorbol ester, PMA [7], or EGF [8] resulted in an augmentation of forskolin-stimulated adenylate cyclase activity in these cells.

The recent recognition of the importance of phospholipase D in signal transduction mechanisms [9,10] prompted us to examine the potential role of the phospholipase D-linked mechanism on the EGF-mediated augmentation of luteal adenylate cyclase activity. We report here that exogenous phospholipase D can enhance the LH-stimulated adenylate cyclase activity and that EGF is in a position to activate the endogenous phospholipase D in luteal cells, and propose that its enzymatic hydrolysis product, phosphatidic acid, may represent a new link between the ligand-activated EGF receptor and the sensitization of the gonadotrophin receptor-linked adenylate cyclase system.

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2. MATERIALS AND METHODS

2.1. *Preparation of cells and incubation procedure*

Bovine luteal cells were isolated and purified as described previously [7,11]. Incubations of cells (200,000) were routinely carried out in 500 μl minimum essential medium containing 25 mM HEPES, pH 7.4, and 0.1% bovine serum albumin (medium) at 37°C for the indicated durations without any addition or with either 10 ng/ml EGF (Boehringer-Mannheim, Mannheim), 10 nM 4β phorbol myristate acetate, PMA (Sigma, Deisenhofen) or 2 U/ml phospholipase D from *Streptomyces chromofuscus* (Sigma), unless otherwise indicated. The cell pellets were stored frozen at −80°C for further analysis.

2.2. *Preparation of the luteal cell membrane fraction*

The cell pellet was thawed and homogenized in a Dounce-type all glass homogenizer in 1 ml 10 mM Tris-HCl, pH 7.4, containing 1 mM dithiothreitol (buffer A) supplemented with the following protease inhibitors (all from Sigma, Deisenhofen): 10 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml amastatin followed by ultrasonication in a Branson Sonifier B12, twice for 10 s each at a power setting 2. Immediately, thereafter sucrose solution was added to a final sucrose concentration of 0.25 mM. The homogenate was centrifuged at 200 × *g* for 15 min at 4°C. The supernatant obtained was recentrifuged at 40,000 × *g* for 60 min at 4°C. The pellet obtained was resuspended in buffer A and was used as luteal membrane fraction. Protein content was determined as described in [12].

2.3. *Adenylate cyclase assay*

The enzyme assay was carried out in a final volume of 100 μl at 36°C for 15 min. The standard incubation mixture [8] consisted of 40 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM 3-isobutyl-1-methyl xanthine, 1 mM ATP, 10 mM creatine phosphate, 13.2 U/ml creatine

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phosphokinase (all reagents from Sigma) and 10 μg protein equivalent of membrane fraction. Where indicated, Forskolin (Calbiochem, Bad Sodem, Germany) or bovine LH (a generous gift from NIH, NIADDK, Bethesda, MD) was added. The incubation was terminated by adding 1 ml ice-cold 100% ethanol. The amount of cAMP formed was measured by a specific radioimmunoassay [7,8].

2.4. *In vitro treatments of luteal membrane fraction*

The membrane fraction (80 μg protein) was incubated in 100 μl PIPES buffer, pH 7.0, in the presence of 10 mM $MgCl_2$, 50 μM $CaCl_2$, 50 μM EGTA, 100 μM ATP, without any other addition or with either 100 μM phosphatidic acid (arachidonic/stearic acid, Sigma, Deisenhofen), 10 ng/ml EGF or 10 nM PMA for 10 min. At the end of the incubation, the tubes were placed in an ice–water bath. The samples were immediately centrifuged at 40,000 × *g* for 60 min. The resulting pellet was resuspended and after determination of its protein content, was used in the adenylate cyclase assay.

2.5. *Lipid analysis*

Cells were labelled for 3 h with 5 μCi [^{3}H]myristic acid (specific activity 54 Ci/mmol, Amersham, Braunschweig) in 500 μl medium and were collected by centrifugation at 100 × *g* for 15 min and washed thrice with 500 μl medium each time. The cells were then loaded for 5 min with 1.5 mM $CaCl_2$ and 50 μM cytochalasin B [13] before adding the agonists. Cells were then stimulated with EGF or PMA for 1 min and the incubation was stopped by placing the tubes in an ice–water bath and rapidly adding 10 μl conc. HCl. The cell extracts were transferred to Eppendorf tubes for lipid analysis [14]. Briefly, the samples were layered with 0.25 ml methanol followed by 0.5 ml chloroform and finally 0.25 ml water was added. The mixture was vortexed vigorously and the phases were separated by centrifugation at 2,000 × *g* for 15 min. The upper phase was removed almost completely and discarded. The lower phase was collected and dried under nitrogen flow. The dried extract was redissolved in minimum amount of $CHCl_3/CH_3OH$ (9:1) and applied to silica gel G 60 thin layer plates (Whatman, LK). The plates were developed in organic phase from: ethyl acetate/isooctane/glacial acetic acid/water (110:50:20:100, v/v) [15] and the spots were located by staining the standards with iodine vapour and autoradiography, for which the plates were prepared for fluorography by spraying with EN3HANCE (DuPont NEN, Bad Homburg, Germany) and then exposed to film at −80°C. For quantitative estimation of ^{3}H incorporation, the silica gel areas of interest were scrapped, placed in scintillation vials, extracted with 1 ml methanol/conc. HCl (150:1) and counted after addition of 9 ml scintillation cocktail.

2.6. *Data presentation*

If not otherwise stated, the results have been presented as mean ± S.E. from quadruplicate determinations from one of three experiments performed.

3. RESULTS

3.1. *Exogenous phospholipase D increases luteal adenylate cyclase activity*

Pretreatment of luteal cells with various concentrations of phospholipase D led to a dose-related increase in basal, forskolin- and LH/GTP-stimulated adenylate cyclase activity measured in membranes prepared from the pretreated cells (Fig. 1). In separate control experiments (data not shown), the effects of phospholipase A2 and C (both phosphatidic choline- and phosphatidic inositol-specific) were examined but neither of these could enhance the adenylate cyclase activity.

Since in luteal cells, the LH receptor-coupled adenylate cyclase signalling pathway is of major physiological relevance and phospholipase D treatment does increase the LH/GTP-stimulated cyclase activity (Fig. 1), in all experiments described below adenylate cyclase has been measured in the presence of LH/GTP.

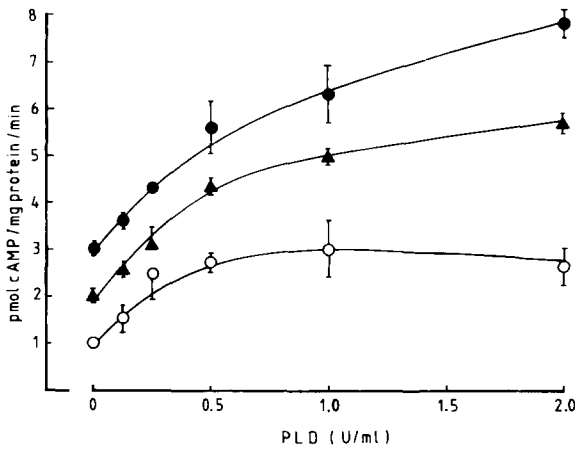

Fig. 1. Exogenous phospholipase D stimulates luteal adenylate cyclase activity. Luteal cells were treated for 10 min with various concentrations of phospholipase D (PLD), following which membrane fractions were prepared for adenylate cyclase assay, carried out for 15 min in the absence of any addition (○) and in the presence of 25 μM forskolin (●) or 100 ng LH plus 1 μM GTP (▲), as described in section 2.

Fig. 2 depicts the time course of the cyclase assay carried out with membranes prepared from untreated or phospholipase D-treated cells. It is evident that the enzyme activity increased linearly with the increase in the duration of incubation, and that, compared to untreated cells, the enzyme activity was markedly greater for the membrane preparation from phospholipase D-treated cells at all time points of the assay. Furthermore, kinetic analysis of the enzyme activity (Fig. 3) revealed

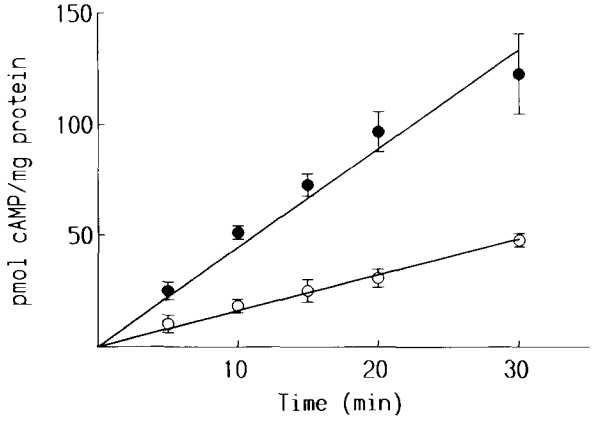

Fig. 2. Time course of adenylate cyclase assay using membranes prepared from untreated and phospholipase D-treated luteal cells. The cells were pretreated for 10 min without any addition (○) or with 2 U/ml phospholipase D (●). The membrane fractions were prepared and used in the adenylate cyclase assay, carried out for various durations in the presence of LH/GTP (see Fig. 1 for other details).

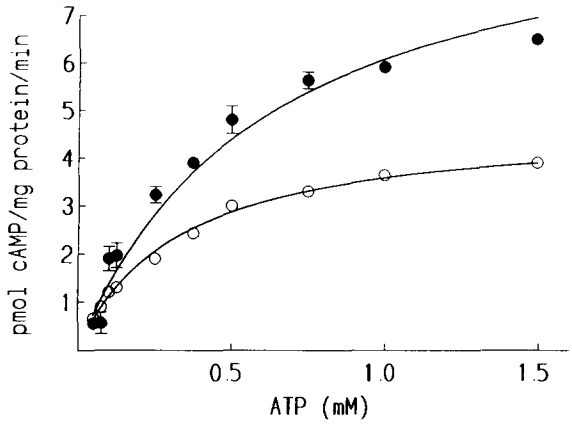

Fig. 3. Effects of phospholipase D treatment on the kinetics of adenylate cyclase activity measured in the presence of LH/GTP. Membrane fraction was prepared from luteal cells treated for 10 min with phospholipase D (2 U/ml). Adenylate cyclase assay was carried out for 15 min in the absence (○) or in the presence (●) of LH/GTP with varying concentrations of the substrate, ATP.

that the preincubation with phospholipase D led to an increase in the V_{max} of the enzyme from 4.1 (control untreated cells) to 9.8 pmol cAMP/mg protein/min (phospholipase D-treated cells) without affecting the K_m value for ATP.

3.2. *Comparison between the effects produced by phospholipase D with those caused by EGF on luteal adenylate cyclase activity*

Further, in terms of physiological relevance, it was of interest to determine whether an exogenous ligand will be in a position to activate the endogenous phospholipase D in luteal cells. In this context EGF was of interest to us, since we have previously characterized the receptors for EGF on these cells and have shown that EGF did augment forskolin-stimulated adenylate cyclase activity [8]. The effect of EGF on LH/GTP-stimulated adenylate cyclase activity in luteal cell has not yet been reported. We show now in Fig. 4, that pretreatment of luteal cells with either 10 ng/ml EGF or 2 U/ml phospholipase D resulted in a comparable augmentation of the enzyme activity measured in the presence of LH/GTP.

3.3. *EGF stimulates phosphatidic acid formation in luteal cells*

Next we assessed whether phospholipase D-mediated phospholipid breakdown could be induced by EGF in luteal cells. In this experiment (Fig. 5), for the sake of comparison, we have also included PMA, since activation of protein kinase C in a variety of cells is known to result in an activation of phospholipase D [16,17] on the one hand and augmentation of adenylate cyclase activity [1–3,7] on the other. We have preincubated the cells with [^{3}H]myristic acid for 3 h and then stimulated with either EGF or PMA for one minute. The formation of [^{3}H]phosphatidic acid was monitored by thin layer chromatographic separation of the products (Fig. 5A) and by counting the radioactivity associated with the silica gel area containing the phosphatidic acid spot (Fig. 5B). It is clear from the data shown, that both EGF and PMA could markedly (4- to 5-fold) increase the formation of phosphatidic acid.

3.4. *Stimulation of luteal adenylate cyclase activity by phosphatidic acid*

We have next incubated the luteal membrane preparation in vitro with phosphatidic acid, a hydrolysis product of phospholipase D and show that phosphatidic acid pretreatment results (Fig. 6A) in an augmentation of the adenylate cyclase activity in a dose-dependent manner, maximum augmentation being achieved with 50–100 μM PA. It is evident from the data shown in Fig. 6B, that 100 μM PA was able to augment adenylate cyclase activity to an extent comparable with that caused by 10 ng/ml EGF or 10 nM PMA.

4. DISCUSSION

We have presented evidence here showing that both exogenous phospholipase D and its hydrolysis product phosphatidic acid are able to augment the gonadotrophin receptor-coupled adenylate cyclase activity in luteal cells. This effect could be mimicked by EGF, which was also shown to be able to stimulate intracellular phosphatidic acid accumulation, as a sequelae to the activation of cellular phospholipase D. As far as we are

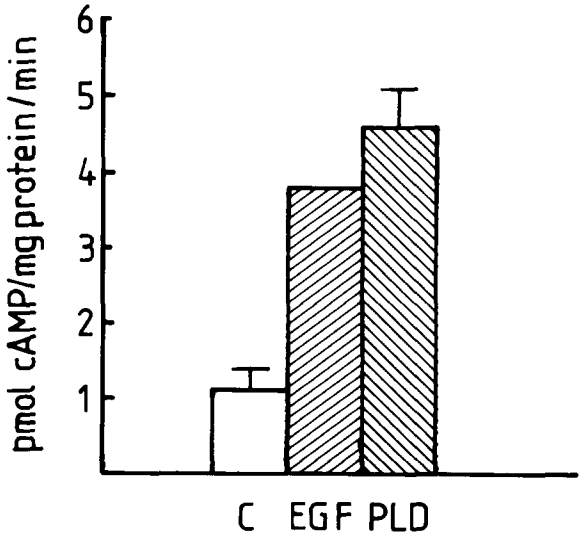

Fig. 4. EGF can mimic the augmentary effects of phospholipase D on LH receptor-coupled adenylate cyclase activity in luteal membrane preparation. The cells were treated for 10 min without (C) any addition or with 2 U/ml phospholipase D (PLD) or 10 ng/ml EGF. The enzyme activity was measured in membrane fractions prepared from the cells.

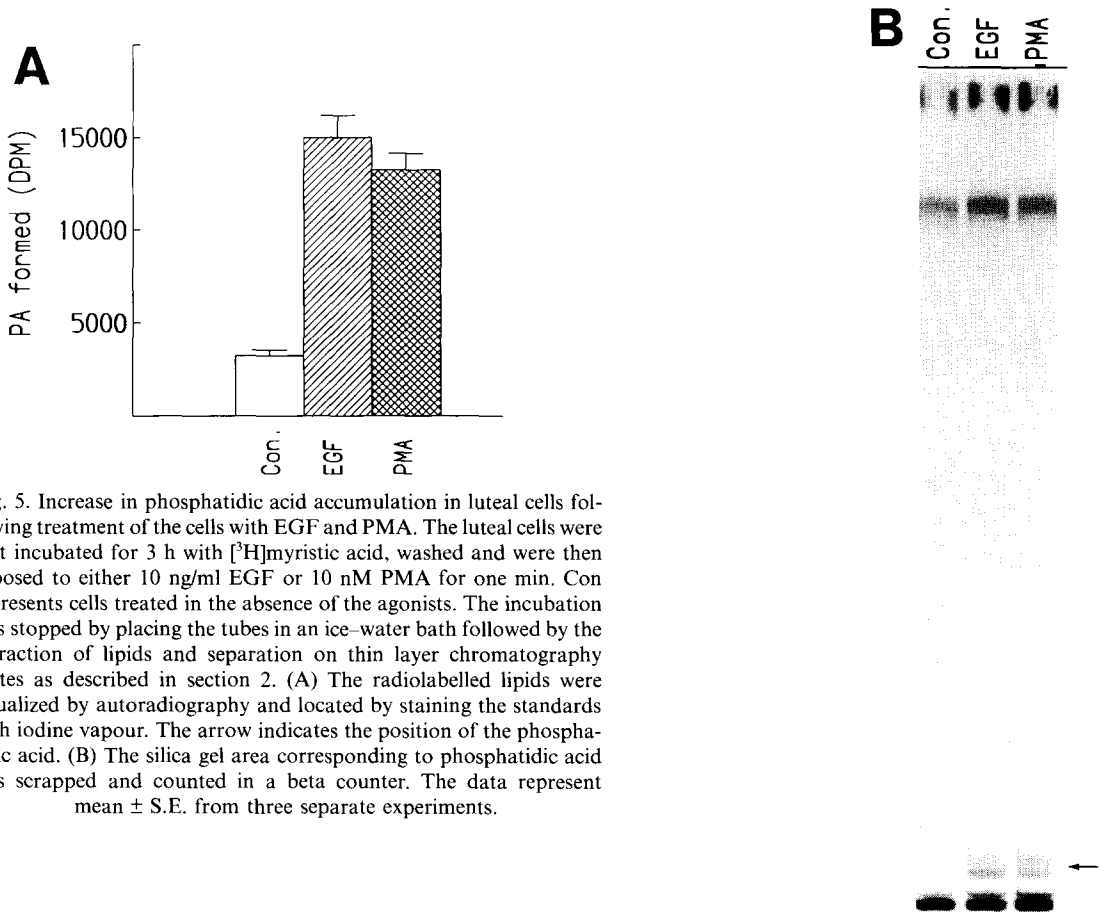

Fig. 5. Increase in phosphatidic acid accumulation in luteal cells following treatment of the cells with EGF and PMA. The luteal cells were first incubated for 3 h with [^{3}H]myristic acid, washed and were then exposed to either 10 ng/ml EGF or 10 nM PMA for one min. Con represents cells treated in the absence of the agonists. The incubation was stopped by placing the tubes in an ice–water bath followed by the extraction of lipids and separation on thin layer chromatography plates as described in section 2. (A) The radiolabelled lipids were visualized by autoradiography and located by staining the standards with iodine vapour. The arrow indicates the position of the phosphatidic acid. (B) The silica gel area corresponding to phosphatidic acid was scrapped and counted in a beta counter. The data represent mean ± S.E. from three separate experiments.

aware, this is the first demonstration linking the phospholipase D activation with a sensitization of a receptor-coupled adenylate cyclase signalling system.

This stimulatory effect of EGF on luteal adenylate cyclase agrees well with the results previously published [8,18,19] and reinforce the emerging concept that a growth factor receptor may crosstalk with G protein-coupled seven-transmembrane-domain receptors linked to cAMP signalling pathway. Also the link between the activation of protein kinase C and the adenylate cyclase system has been documented in luteal [7] and other cells [1–3]. It appears that in luteal cells at least two signals are required for full activation of the gonadotrophin receptor-coupled adenylate cyclase. The first signal, for example EGF in this case, activates a phospholipase D pathway resulting in the formation of phosphatidic acid which in turn sensitizes the adenylate cyclase system for an optimal stimulation through the second signal, LH.

The activation of phospholipase D by EGF and PMA as observed in luteal cells, has been well documented in a variety of other cells [16,17,20,21]. However, our results differ from previous observations in other cell types [22,23], where the products of phospholipase D were reported to have an inhibitory influence on adenylate cyclase activity. This difference may be explained by the fact that such effects vary in a cell- or tissue-specific manner. For example, recently evidence has indeed become available showing that the manner in which the activation of protein kinase C would affect the adenylate cyclase activity in a particular cell type depends upon the specific types of adenylate cyclase present in that particular cell [24,25].

In conclusion, we propose that the phosphatidic acid accumulated as a result of the activation of phospholipase D, after treatment of the cells with EGF or PMA, serves as an intracellular second messenger which is able to upregulate the LH receptor-coupled adenylate cyclase in luteal cells. A second messenger [26] and growth factor-like [27] role has been previously proposed for phosphatidic acid; however, its role as a link between the signalling mechanisms associated with the single transmembrane domain tyrosine kinase-linked receptor and the seven transmembrane domain adenylate cyclase-linked receptors has not been previously recognized. Whether phosphatidic acid itself modulates the adenylate cyclase or its effect is mediated via a specific kinase, as suggested recently [28], remains the subject for future investigation.

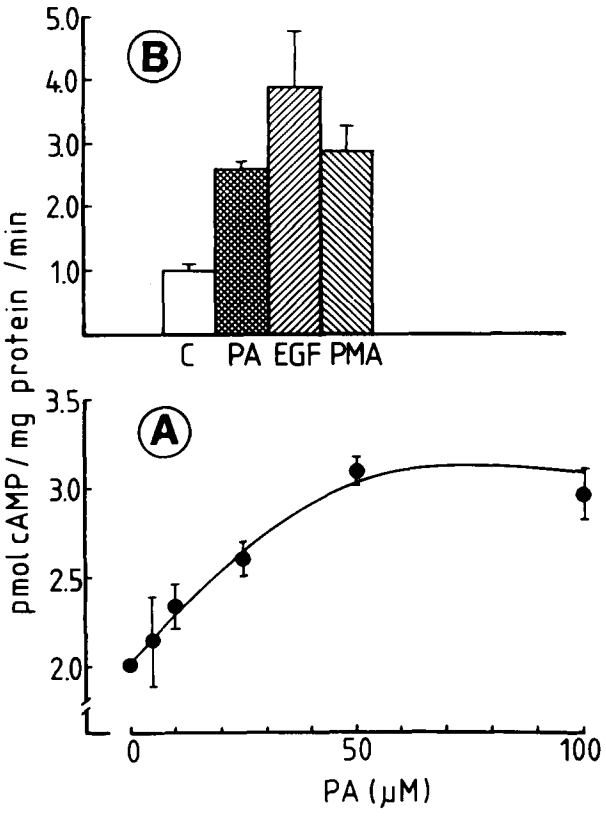

Fig. 6. Prior treatment of luteal membranes with phosphatidic acid leads to a stimulation of adenylate cyclase. (A) The membrane fractions were incubated as described in section 2 with various concentrations of phosphatidic acid (PA) for 10 min. The incubation was stopped by placing the tubes in an ice–water bath. The samples were immediately centrifuged at 40,000 × *g* for 60 min at 4°C. The resulting pellets were used for adenylate cyclase assay in the presence of LH/GTP. (B) The membrane fraction was incubated without any addition (C) or with either 100 μM phosphatidic acid (PA), 10 ng/ml EGF or 10 nM PMA and assayed for adenylate cyclase activity as above.

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