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Original article

Effect of growth factors on proliferation, apoptosis and protein kinase A expression in cultured porcine cumulus oophorus cells

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Abstract — The proliferation, apoptosis and protein kinase A (PKA) in porcine cumulus oophorus (CO) before and after 40 h of culture together with oocytes in the presence of IGF-I, IGF-II and EGF (all at 10 ng·mL^{-1} medium) were compared. Cellular proliferation, apoptosis and PKA contents were evaluated by immunocytochemistry using specific antibodies against PCNA, TUNEL and catalytic (C-alpha) and regulatory (RI) subunits of PKA. The in-vitro culture of oocyte-CO complexes in a basal medium was accompanied by a decrease in the proportion of PCNA-positive CO cells (from 51 to 36%, $p < 0.05$). The addition of either IGF-I or EGF to the culture medium prevented this process and increased the proliferation rate (64 and 67% respectively, $p < 0.001$). During culture, the percentage of apoptotic (TUNEL-positive) CO cells increased from 42 to 57% ($p < 0.01$). The addition of IGF-I or EGF resulted in the inhibition of apoptosis to 36 and 12% respectively ($p < 0.001$). IGF-II and EGF reduced the amount of PKA catalytic subunits in the CO (percentage of cells with immunoreactive PKA catalytic subunits (28%, $p < 0.05$ and 27%, $p < 0.05$ respectively; versus control –41%), whilst the effect of IGF-I on this index was insignificant (31%). The expression of the PKA regulatory subunit was increased by EGF (51% compared with 29% in the control, $p < 0.05$), but not by IGF-I or IGF-II (30 and 29%).Our observations demonstrate that 40 h of culture of porcine CO resulted in a decrease in the proliferation and development of apoptosis in CO cells. IGF-I or EGF can stimulate proliferation and inhibit apoptosis. The influence of growth factors on the PKA content of the CO suggests that cAMP/PKA may be a mediator of the action of growth factors on these cells. The differential effects of IGFs and EGF on the regulatory subunit of PKA may indicate differences between their mechanisms of action.

insulin-like growth factor I / insulin-like growth factor II / epidermal growth factor / cumulus oophorus / proliferation / apoptosis / protein kinase A

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Résumé — Effets des facteurs de croissance sur la prolifération, l'apoptose et l'expression de la protéine kinase A des complexes « cumulus-ovocyte » de porc en culture. Des complexes « cumulus-ovocyte » de porc (COC) ont été mis en culture en présence d'IGF-I, IGF-II, EGF (10 ng·mL^{-1} de milieu). Leurs effets sur la prolifération cellulaire, l'apoptose et la protéine kinase A (PKA) ont été évalués par immuno-cytochimie à l'aide d'anticorps spécifiques dirigés contre PCNA, TUNEL et les sous-unités catalytique (C-alpha) et régulatrice (RI) de PKA. En milieu témoin, les cellules des COC montrent une diminution de prolifération (de 51 à 31 % de cellules PCNA positives, $p < 0,05$). Avec IGF-I ou EGF dans le milieu, on constate au contraire une augmentation de la prolifération par comparaison aux niveaux de départ (64 et 67 % respectivement, $p < 0,001$). Pendant la culture, les cellules apoptotiques (TUNEL-positives) ont augmenté de 42 à 57 % ($p < 0,01$) ce qui est inhibé par l'addition d'IGF-I ou d'EGF (36 et 12 % des témoins respectivement, $p < 0,001$). IGF-II et EGF ont réduit de façon significative les quantités de la sous-unité catalytique de PKA (cellules immuno-détectables pour cette sous-unité : 28 et 27 % respectivement par comparaison aux témoins : 41 %, $p < 0,05$) alors que l'effet d'IGF-I n'a pas été significatif (31 %). EGF a provoqué une augmentation de la sous-unité régulatrice de PKA (51 % versus 29 % chez les témoins, $p < 0,05$) alors que IGF-I et IGF-II ont été sans effet sur cette dernière (30 et 29 % respectivement). Nos observations montrent que les cellules de COC de porc ont une activité de prolifération en diminution et une apoptose en augmentation lorsqu'elles sont cultivées pendant 40 h. IGF-I et EGF peuvent stimuler la prolifération et inhiber l'apoptose. L'influence de ces facteurs de croissance sur PKA suggère que cAMP/PKA peut être un médiateur de ces facteurs sur ces types cellulaires. Les effets différenciés des IGFs et de l'EGF sur les sous-unités de PKA peuvent indiquer des différences de leurs modes d'action.

IGF-I / IGF-II / EGF / cumulus oophorus / prolifération / apoptose / protéine kinase A

1. INTRODUCTION

The cumulus oophorus (CO) is important in the control of oocyte maturation [2, 4]. In particular, it can delay [8, 17, 18] or advance [23, 26] porcine oocyte maturation. The effect of the CO may be due to the local production of glycosaminoglycans, which facilitate the maturation of the oocyte-CO complex [2, 4, 24], or to steroidogenesis which is also involved in the control of oocyte maturation [11, 22]. Cumulus and/or other follicular cells can also influence the oocyte through the local production of insulin-like growth factors type I or II (IGF-I, IGF-II) or epidermal growth factor (EGF). These are potent regulators of proliferation, apoptosis and steroid hormone secretion by ovarian cells [1, 5, 13, 15, 29] but also stimulate oocyte maturation [10, 11, 20, 21, 26, 28, 31]. Growth factors may act on the oocyte via the CO: the absence of a CO was found to prevent the maturational effect of IGF-I on bovine [10] and rabbit [11] oocytes, although in other species (murine [3], porcine [25]) such dependence was not seen.

The actions of growth factors on the CO itself are poorly understood. EGF is able to stimulate cumulus expansion around porcine [20] and bovine [10] oocytes. Harper and Brackett [7] reported the stimulation of bovine CO expansion by IGF-I but others [10] did not observe this effect. There are reports that IGF-I can stimulate [H]^3-thymidine incorporation and progesterone secretion by bovine CO [31] and the release of androstenedione and estradiol by rabbit CO [11]. Thus, information concerning the influence of growth factors on CO is limited and uncertain. The actions of other growth factors, especially IGF-II and EGF, on the proliferation and apoptosis in CO have not been studied.

The intracellular mechanisms of action of growth factors on the CO are unknown as well. Some substances can affect cell

functions via changes in the formation of cAMP, which induces the production of protein kinase A (PKA) and the dissociation of PKA holoenzyme forming the catalytic and regulatory subunits. The catalytic subunit can translocate into the nucleus and via CREB and other transcription factors affect expression of particular genes [6]. There is some evidence that effects of growth factors on ovarian cells can be mediated by the cAMP/PKA system. In particular, IGF-I stimulates cAMP formation in rabbit granulosa cells [13], porcine follicles [27] and oocytes [20]. IGF-I and IGF-II, but not EGF, increase the PKA content of rabbit granulosa cells [13] and porcine oocytes [28]. In the rabbit, PKA blockers prevent IGF-I from affecting proliferation, steroid hormone secretion by granulosa cells and embryogenesis [13]. They also inhibit the IGF-induced stimulation of oxytocin release [14] and apoptosis [12] by bovine granulosa cells. Similarly PKA blockers prevent IGF-I, IGF-II [28] and EGF [20, 28] from stimulating porcine oocyte maturation. Therefore, cAMP/PKA is a potential mediator of growth factor action on various ovarian cells.

The aims of our investigation were: (1) to examine CO proliferation and apoptosis during in-vitro maturation, (2) to detect the presence of catalytic and regulatory subunits of PKA in the CO, and (3) to determine the influence of IGF-I, IGF-II and EGF on these processes.

2. MATERIALS AND METHODS

2.1. Processing and culture of oocyte-CO complexes

Ovaries without visible abnormalities were removed from non-cycling Slovakian white gilts, 6 months of age, within two hours after killing at a local abattoir and transported to the laboratory at +4 °C. Oocyte-CO complexes were isolated and processed as described previously [28]. Briefly, the oocyte-CO complexes were aspirated by syringes from ovarian follicles 2–8 mm in diameter. Haemorrhagic, cystic, presumptive preovulatory follicles (more than 8 mm) and follicles with visible signs of atresia were excluded. The aspirates were washed 3 times in TCM-199 (Sigma, St. Louis, USA) supplemented with 10% FCS (Sigma). Oocyte-CO complexes were cultured in 5% CO_2 in the air, at 37.5 °C under paraffin oil in 1 mL of TCM-199 supplemented with 10% heat inactivated FCS and the 0.5% antibiotic-antimycotic solution (Sigma). Recombinant human IGF-I (Calbiochem, Lucerne, Switzerland; 10 ng·mL^{-1}), IGF-II or EGF (Sigma; 10 ng·mL^{-1}) were added to the medium of the experimental groups. The control medium contained no exogenous growth factors. Oocyte-CO complexes were collected for analysis before and after 40 hours of culture (sufficient time for the completion of maturation in 50–70% of oocyte-CO complexes; [28]). Collection of cells before their full maturation (≤ 48 hours of culture, [28]) was to allow the expression of the stimulatory influence of growth factors on this process.

2.2. Analysis of CO

The oocyte-CO complexes were washed for 1 min in 0.9% Na citrate, CO were isolated using a Pasteur pipette, placed on microscopic slides, fixed with a mixture of pure methanol and glacial acetic acid (3:1) and air-dried.

PKA was detected in all the groups of CO by immunocytochemistry [16] as described previously [23, 28] using primary mouse monoclonal antibodies against the regulatory subunit RI (dilution 1:1 000) and catalytic subunit C-alpha (dilution 1:250; Transduction Laboratories, Lexington, USA). These antibodies cross react with the related subunits of human, dog and rat PKAs. Prior to the experiment, their activity and cross-reactivity at these dilutions with porcine material were confirmed by Western blotting and immunocytochemical analysis

of porcine granulosa cells (not shown). For visualization of PKA, a secondary polyclonal antibody against mouse IgG labeled with horseradish peroxidase (SwAM Px, Sevac, Prague, Czech Republic; dilution 1:500) and DAB-reagent (Boehringer Mannheim GmbH, Mannheim, Germany; 0.1%) was used.

The proliferative activity of CO cells cultured without and with IGF-I and EGF (but not with IGF-II) was evaluated according to the presence of PCNA (Proliferating Cell Nuclear Antigen) in the cells by immunocytochemistry [16] as described previously [13] using anti-PCNA mouse monoclonal antibody (Calbiochem-Novabiochem, La Jolla, USA) in a 1:100 dilution. This antibody cross-reacts with human, mouse, rat and yeast PCNA; its cross-reactivity with porcine material was confirmed by Western blotting (not shown). Visualization was done as described for PKA.

Apoptotic cells in these groups of CO were detected immunocytochemically using the TdT-mediated dUTP-biotin nick end labeling (TUNEL) method (In Situ Cell Death Detection Kit, POD, Boehringer Mannheim GmbH) as described previously [23].

CO cells treated with secondary antibodies and DAB, omitting primary antibodies, were used as a negative control in every immunocytochemical analysis. The presence of substances in the cells and the proportion of PCNA-, TUNEL- and PKA-positive cells in each control and experimental group of COs was determined by light microscopy using a Jenaval (Carl Ceiss, Jena, Germany) microscope.

2.3. Statistics

Each experimental group in one experiment was represented by 4000–6000 cells from 15–45 CO. Before the experiments, CO with the different number and state of cells were distributed approximately equivalently between the groups. The data shown are the means obtained in 3 replicate experiments (3–5 replicates per experiment) performed on separate days using separate pools of cells. Significant differences in the proportion of CO cells containing PKA, PCNA or TUNEL immunoreactivity were evaluated by the Wilcoxon-Mann-Whitney and Dunkan multiple range tests [9]. Differences compared with the control wit $p < 0.05$ were considered as significant.

3. RESULTS

It was observed that CO cells contained PCNA, TdT/TUNEL, catalytic and regulatory subunits of PKA. A regulatory subunit of PKA was observed mainly in the extranuclear region of the cells, whilst the catalytic subunit, PCNA and TdT/TUNEL were localized mainly in the nucleus.

Fifty-one per cent of the cells collected from CO before in-vitro culture contained visible PCNA, whilst after 40 h culture without treatments the proportion had decreased significantly to 36% ($p < 0.001$). The addition of IGF-I or EGF significantly increased the proportion of proliferating cells to 64 and 67% respectively (both $p < 0.001$ compared with the control, culture without additions; Fig. 1). The effect of IGF-II on cell proliferation was not studied in these experiments.

Prior to culture, 42% of the CO cells showed signs of apoptosis (TUNEL positive). After culture, this value rose to 57% ($p < 0.01$). After culture in the presence of IGF-I or EGF, the percentage decreased to 36 and 12% respectively (both $p < 0.001$ with control; culture without additions; Fig. 2). The effect of IGF-II on this process was not evaluated.

The amount of PKA in the CO cells before culture was not analyzed. After culture, the catalytic subunit of PKA was detected in 42% of the CO cells. After

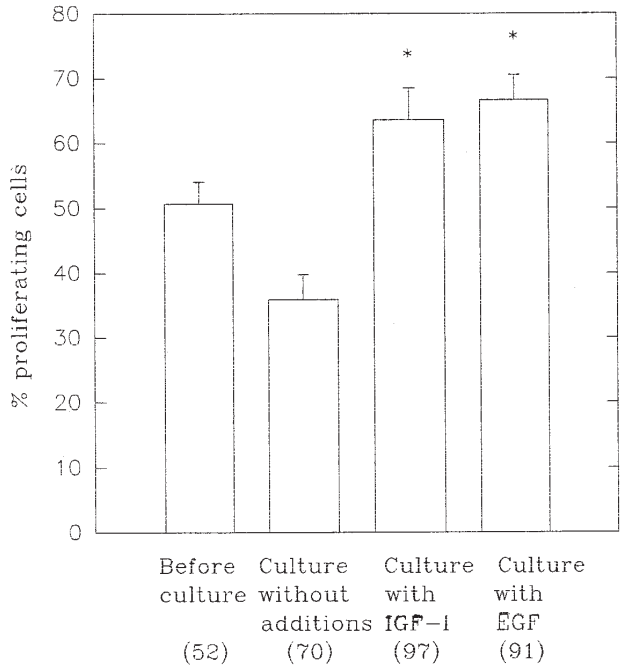

Figure 1. Proliferative activity of porcine CO cells (PCNA test) before and after 40 h of culture with and without growth factors. Values are mean percentages of cells containing specific immunoreactivity ± S.E.M. * Significant ($p \leq 0.05\%$) differences with control (CO cultured without growth factors). The total number of CO analysed is indicated in brackets.

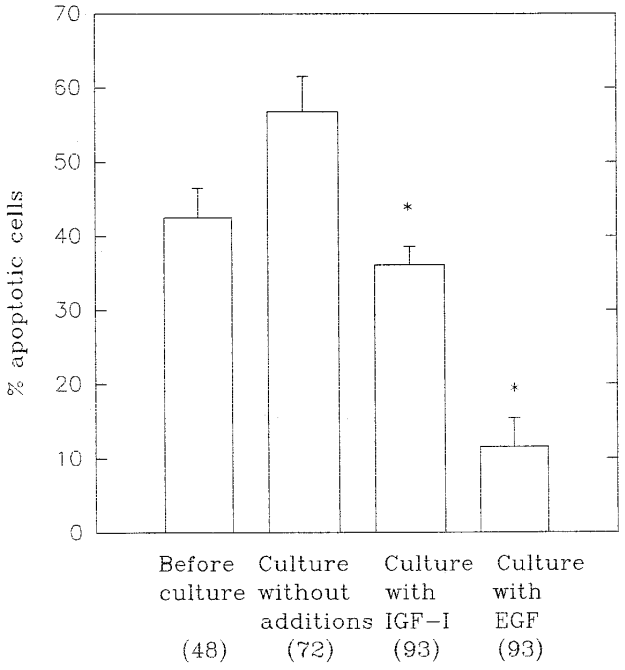

Figure 2. Rate of apoptosis in porcine CO cells (TUNEL method) before and after 40 h of culture with and without growth factors. Values are mean percentages of cells containing specific immunoreactivity ± S.E.M. * Significant ($p \leq 0.05\%$) differences with control (CO cultured without growth factors). The total number of CO analysed is indicated in brackets.

culture in the presence of IGF-II or EGF this percentage decreased significantly to 28% ($p < 0.05$) and 27% ($p < 0.05$) respectively. The suppressive effect of IGF-I on this process was insignificant (to 31%) (Fig. 3a). The regulatory subunit of PKA was detected in 29% of the control cells. The addition of IGF-I or IGF-II did not change this proportion substantially (30 and 29% respectively), but EGF significantly

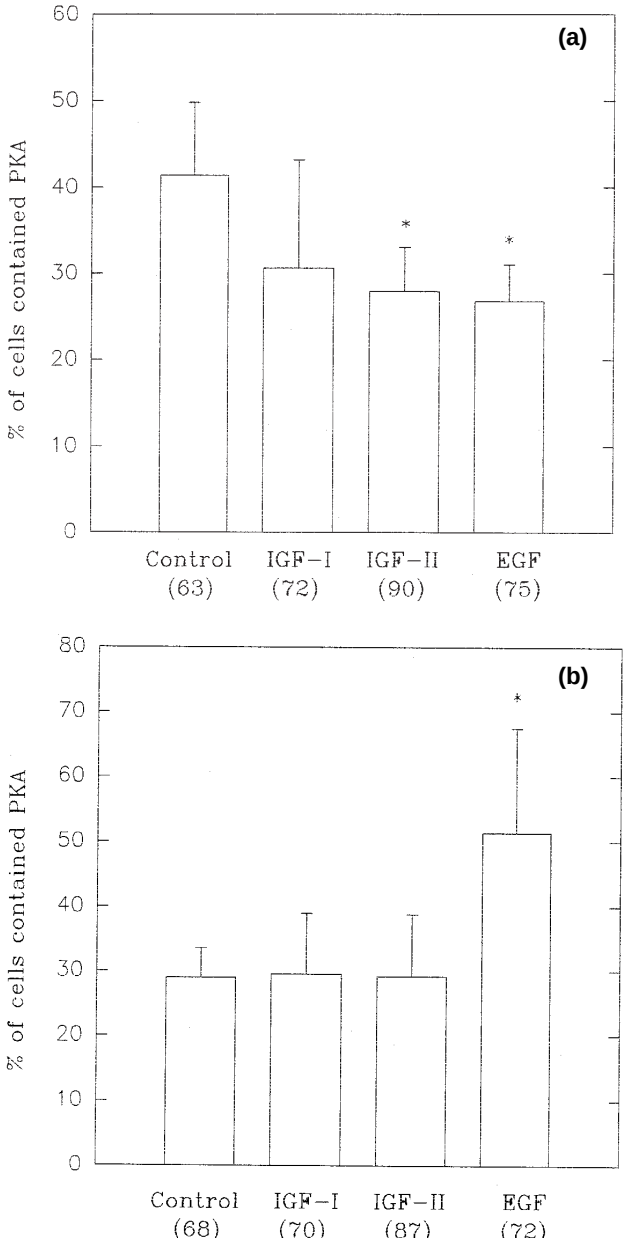

Figure 3. Effect of growth factors on the content of catalytic (**a**) and regulatory (**b**) subunits of PKA in porcine CO cells after 40 h of culture with and without growth factors. Values are mean percentages of cells containing specific immunoreactivity ± S.E.M. * Significant ($p \leq 0.05$) differences with control (CO cultured without growth factors). The total number of CO analysed is indicated in brackets.

elevated the proportion of CO cells with detectable regulatory PKA subunits to 51% ($p < 0.05$; Fig. 3b).

Visible (2–3 times) expansion of CO during the culture both in the absence or presence of growth factors was observed. No significant effect of the growth factors on this parameter was found in our experiments (not shown).

4. DISCUSSION

Our observations of PCNA and TUNEL immunoreactivity suggest that during in-vitro maturation of the oocyte-CO complex, the proliferative activity of the CO declines whilst the apoptotic process develops. These changes may reflect possible degenerative processes within the CO during culture.

The demonstration of the effects of growth factors observed in our experiments suggests that, despite the potential presence of the growth factor in the serum-supplemented medium, additional exogenous growth factors can affect proliferation, apoptosis and PKA in CO, and that growth factors therefore can be involved in the physiological control of these processes. Our detection of IGF-I-induced PCNA accumulation is consistent with the earlier report of Xia et al. [31] on IGF-I-stimulated $[H]^3$-thymidine incorporation in porcine CO. This suggests that IGF-I stimulates the proliferation of CO cells. Our observations of a stimulatory action of IGF-I, IGF-II and EGF on PCNA and of their inhibitory effect on TdT correspond with reports of mitogenic and anti-apoptotic actions of IGF-I and EGF in ovarian granulosa cells [1, 15]. This is the first data to show that proliferation in CO can be regulated not only by IGF-I, but also by IGF-II and EGF, and that growth factors can control apoptosis in CO.

PKA may be involved in the control of CO function and/or oocyte maturation as demonstrated by significant changes in the PKA content of porcine CO (present data) and oocytes [28]. Evidence for this also comes from data on the formation, transport and action of cAMP and PKA, and on the action of cAMP or its analogues on meiosis in murine, bovine and porcine oocyte-CO complexes [4, 18, 21, 24, 28].

The cAMP/PKA system may be a possible mediator of growth factor action on the CO. This hypothesis is supported by the presence of both PKA subunits (RI and C-alpha) in the CO, as well as by significant effects of growth factors on their content. The main target for the action of growth factors appears to be the catalytic subunit of PKA because IGF-II and EGF decreased its content. The amount of the regulatory subunit was unaffected by IGF-I and IGF-II, although EGF caused an increase. The effects of growth factors predominantly on catalytic rather than on the regulatory PKA subunit in CO observed in the present experiment coincides with the effects of GH on PKA subunits in follicular cells [23] and with the influence of growth factors mainly on the PKA catalytic subunit in porcine oocytes [28] reported previously. This suggests the existence of a universal mechanism of action of GH/growth factors on the ovaries, which mainly includes the cAMP/PKA catalytic subunit, but much lesser the cAMP/PKA regulatory subunit. This should be expected because the activation of PKA results in the dissociation of catalytic and regulatory PKA subunits; only the catalytic PKA subunit is biologically active (it enters the nucleus and activates the phosphorylation of transcription factors), whilst the free regulatory subunit is degrading [19]. On the contrary, the stimulatory effect of EGF on the regulatory PKA subunit suggests the involvement of this subunit in the mediation of the EGF effect or on the existence of feedback interrelationships between EGF, and regulatory and catalytic PKA subunits. Qualitative differences in the actions of IGFs and EGF on the regulatory subunit of PKA, as well as significant quantitative differences in their ability to prevent apoptosis may indicate some

differences in the mechanisms of action of different growth factors which require further studies.

These are the first data on the presence of PKA in CO and on its control by growth factors. Why growth factors stimulate PKA in oocytes but inhibit it in CO remains unknown but this may reflect the different roles of growth factors or cAMP/PKA within the different cells. Functional coupling between the oocyte and CO cAMP/PKA systems is also possible. Such coupling has been suggested previously in murine oocyte-CO complexes [4] where cAMP (an activator of PKA) produced in the CO and transported to the oocyte was shown to be a physiological meiosis-inhibiting factor. Moreover, a difference in the pattern of localisation and possible effects of cAMP in follicular cells and oocytes was also previously demonstrated [30]. In our experiments on porcine oocyte-CO complexes, a pharmacological blockade of PKA, using either the cAMP antagonist Rp-cAMP or the direct PKA blocker KT5720, prevented IGF-I, IGF-II and EGF from stimulating porcine oocyte maturation [28]. In addition, the action of in-vitro culture [30] and IGF-I [13] not only on PKA, but also on adenyl cyclase/phosphodiesterase activities resulting in the modulation of the cAMP level was also reported. Thus, in-vitro culture and growth factors could potentially affect oocyte-CO complexes through two pathways, which include adenylate cyclase/phos-phodiesterase/cAMP and cAMP/PKA. The role of the cAMP/PKA system in the control of CO and oocytes requires further study. In this respect, the understanding of the interrelationships between cAMP/PKA, growth factors, oocyte and CO in the control of oocyte maturation and the subsequent embryo production could be important from both theoretical and practical viewpoints. Nevertheless, our observations suggest the changes in proliferation, apoptosis and expression of PKA in porcine CO during culture, induces the maturation of the CO/oocyte complex, and involves IGF-I, IGF-II and EGF in the control of these processes. The involvement of cAMP/PKA-dependent intracellular mechanisms in the mediation effect of growth factors on CO is supposed.

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