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Protein kinases and protein synthesis are involved in desensitization of the plasminogen activator response of rat Sertoli cells by follicle-stimulating hormone

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Abstract Tissue-type plasminogen activator (tPA) secretion is a specific response of Sertoli cells to follicle-stimulating hormone (FSH), which is lower after preincubation of the cells with low FSH concentrations because of FSH receptor/G_s protein uncoupling. In this report, we present evidence that this desensitization induced by the lowest FSH concentrations is suppressed by specific peptidic inhibitors of endogenous PKA and PKC in permeabilized Sertoli cells. In contrast, desensitization promoted by slightly higher FSH concentrations is not mediated through PKA or PKC activation but is dependent on protein neosynthesis.

Key words: Desensitization; Protein kinase cAMP dependent; Protein kinase C; Tissue-type plasminogen activator; Follicle-stimulating hormone; Sertoli cell; Rat

1. Introduction

Hormone stimulation usually leads to subsequent desensitization of their target cells towards the same hormone (homologous desensitization) and sometimes also towards other hormones (heterologous desensitization). These alterations of cell responsiveness are mediated by a combination of different mechanisms occuring over different time scales, ranging from seconds to days. Two main forms of cell desensitization have been identified at the level of the first steps of the hormonal transduction pathway. Slow mechanisms (hours to days) involve alteration in receptor synthesis and degradation. This degradation after internalization is known as down-regulation and is provoked by rather high concentrations of hormone. Rapid mechanisms (seconds to hours) include functional uncoupling between receptors and the G_s proteins, usually due to phosphorylation of one or both of these molecules.

We have previously demonstrated [1] that low follicle-stimulating hormone (FSH) concentrations (0.3–5 ng/ml) inhibit by 70% the subsequent tissue-type plasminogen activator (tPA) response of Sertoli cells to a submaximal dose of hormone (100 ng/ml) over a time lag of 2.5 h. This desensitization process implies uncoupling of the FSH receptor and the G_s protein, the likely site of the lesion being the FSH receptor. This uncoupling is in agreement with several previous reports on the desensitization by FSH of Sertoli cell responses [2–5].

Desensitization of Sertoli cell tPA response can also be induced, but to a weaker extent, by another ligand such as the β-adrenergic agonist, isoproterenol, as well as by cAMP analogs (PKA activators) or phorbol esters (PKC activators).

By cross-checking all these observations, we hypothesize that two different mechanisms might be involved in FSH-induced desensitization of tPA response: first, one triggered by extremely low FSH concentrations, by isoproterenol as well as protein kinase activators; and second, one induced only by slightly higher concentrations of FSH. These two mechanisms are referred to as heterologous and homologous desensitization, respectively.

In the present work, we have assessed the roles of PKA and PKC and protein synthesis in FSH-induced desensitization of the tPA secretion of rat Sertoli cells in vitro, in order to test this hypothesis.

2. Materials and methods

2.1. Materials

Cycloheximide, phorbol 12,13-dibutyrate (Pdbu), dibutyryl-cAMP (db-cAMP) and Soybean trypsin inhibitor were purchased from Sigma Chemical Co. (St. Louis, MO). Digitonin and Leibovitz's L15 medium were obtained from Serva (Heidelberg, Germany). PKC inhibitor, PKCI (19–36) was obtained from Neosystem (Strasbourg, France), and PKA inhibitor (PKAI), PKI (6–22) amide from Gibco BRL. Equine FSH (eFSH) (CY1368: 864 × NIH-FSH-S1 in homologous equine RRA) was purified in our laboratory [6].

2.2. Isolation of rat seminiferous tubule cells

Rat seminiferous tubule cells were prepared from 16 to 18-day-old rat testes (Wistar INRA 03) according to the method of Dorrington et al. [7] with the following modifications. Collagenase digestion was omitted and replaced by an additional mechanical dispersion performed just after inhibition of trypsin activity by a specific trypsin inhibitor (Soybean). Tubular cell preparations contained more than 75% Sertoli cells, about 17 ± 5% germ cells and 4 ± 2% myoid cells [8]. Identification of germ cells was achieved by staining with Trioxy hematein ferric and that of myoid cells by the alkaline phosphatase reaction [9].

2.3. Incubation of Sertoli cells and cell permeabilization

Incubations of 0.5×10^6 cells were performed in 300 μl of L15 medium and in 3 ml polystyrene tubes. The cells were preincubated for 2.5 h in the presence of the desensitizing agents, then washed, and finally incubated for 4 h at 34°C in the presence of a submaximal dose (100 ng/ml) of FSH. The activity of secreted tPA in the media was determined as previously described [10]. In the permeabilization experiments, digitonin was added 10 min before the preincubations with the desensitizing agents and was present during the 2.5 h preincubation period. It was removed during washings before the subsequent 4 h stimulation by FSH. The digitonin concentration required to permeabilize the cells was adapted in each experiment by increasing its concentration until more than 95% of the cells were positive in the Trypan blue

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test. The different inhibitors were added to the cells 10 min before the addition of FSH for the preincubation.

2.4. Assay of tissue type plasminogen activator activity

The tPA content of incubation media was assayed by the ^{125}I-labeled fibrin digestion method as previously described [11]. The tPA concentration promoting the conversion of plasminogen to plasmin was determined from the release of radioactivity into the medium during fibrinolysis of insoluble radiolabeled fibrin by plasmin. Then, tPA activity was expressed as human tPA units, as determined with reference to a standard preparation of human tPA [12]. We have previously demonstrated the absence of tPA inhibitor in the medium of cells preincubated in the presence or absence of FSH [1].

2.5. Statistics

Statistical analyses of the data were performed using a single mean Student's *t*-test (Statview, Abacus Concepts, CA, USA), with $P < 0.05$ considered to be statistically significant. All experiments were repeated at least three times.

3. Results

3.1. Permeabilization of Sertoli cells

In order to render Sertoli cells permeable to molecules such as peptides of approximately 2,000 Da, the digitonin permeabilization method was tested. The susceptibility of Sertoli cells in suspension to digitonin permeabilization was studied by scoring Trypan blue-positive cells as a function of digitonin concentration. A good permeabilization, assessed by more than 95% of Trypan blue-stained Sertoli cells, required a digitonin concentration of about 0.006% for a Sertoli cell suspension at a density of $3 \cdot 10^6$ cells per ml. At digitonin concentrations higher than 0.012%, cell lysis was observed.

The effect of 0.006% digitonin treatment on Sertoli cell biological function was studied further. Fig. 1 shows that digitonin-treated cells can be stimulated by FSH, with no change in the pattern of desensitization of the tPA response compared to control cells (not permeabilized). In both intact and permeabilized Sertoli cells, FSH concentrations from 0.3 to 5 ng/ml desensitized the subsequent tPA production induced by 100 ng/ml FSH, with a maximum inhibition of 60 ± 5% at 5 ng/ml FSH.

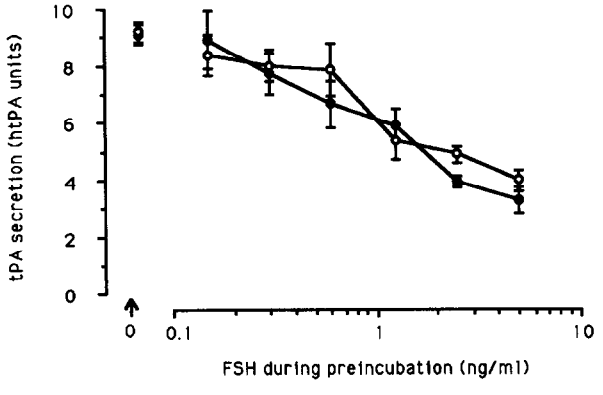

Fig. 1. Effect of cell permeabilization by digitonin on FSH stimulation and FSH-induced desensitization of tPA secretion. Sertoli cells were exposed (●) or not (○) to digitonin for 10 min and subsequently preincubated for 2.5 h with the indicated concentrations of FSH. After a wash, Sertoli cells were then incubated for 4 h with FSH (100 ng/ml) and tPA levels were determined. Values are means ± S.E.M. of triplicate determinations of a representative experiment.

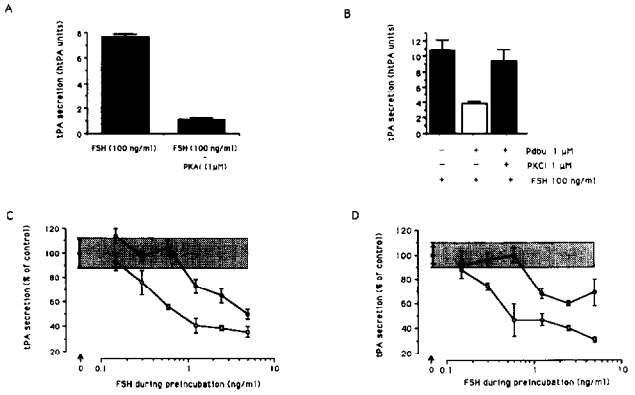

Fig. 2. Effect of inhibitors of protein kinases on FSH stimulation and desensitization of tPA secretion by FSH in permeabilized rat Sertoli cells. (A) Effect of PKAI on FSH stimulated production of tPA. Permeabilized Sertoli cells were preincubated for 10 min in the presence (hatched bar) or absence (black bar) of 1 μM PKAI, subsequently stimulated for 4 h with 100 ng/ml FSH and tPA levels were determined. Values are means ± S.E.M. of triplicate determinations of a representative experiment. (B) Effect of PKCI on PKC activity. Permeabilized Sertoli cells were preincubated for 10 min in the presence or absence of 1 μM Pdbu and/or 1 μM PKCI, subsequently stimulated for 4 h with 100 ng/ml FSH and tPA levels determined. Values are means ± S.E.M. of triplicate determinations of a representative experiment. (C and D) Effect of PKAI and PKCI on FSH desensitization. Permeabilized Sertoli cells were first incubated for 10 min in the presence (●) or absence (○) of 1 μM PKAI (C) or 1 μM PKCI (D). Then, FSH was added at the indicated concentrations and preincubations were continued for 2.5 h. After washing, the cells were then incubated for 4 h with 100 ng/ml FSH. tPA levels were determined and expressed as a percentage of the tPA production in the absence of FSH during the 2.5 h preincubation and with or without the protein kinase inhibitor used. Values are means ± S.E.M. of triplicate determinations of a representative experiment.

3.2. Effect of peptidic inhibitors of protein kinases A and C

First, we verified the effectiveness and specificity of the peptide inhibitors of the protein kinases (Fig. 2A and B) in permeabilized Sertoli cells stimulated for 4 h with 100 ng/ml FSH.

Fig. 2A shows that 1 μM of the PKA inhibitor (PKAI) abolished the FSH-stimulated tPA secretion, confirming the involvement of PKA in this action. The PKC inhibitor (PKCI) specificity was tested through its capacity to reverse the inhibitory effect of a PKC activator, the phorbol ester Pdbu (Fig. 2B). PKC activation by 1 μM Pdbu in Sertoli cells resulted in a 60% decrease in the tPA secretion induced by 100 ng/ml FSH. When PKCI (1 μM) was added together with Pdbu, the tPA secretion induced by 100 ng/ml FSH was restored, demonstrating its inhibitory activity on PKC activity in permeabilized Sertoli cells.

Preincubation of the cells with PKAI provoked a shift in the FSH doses required for the appearance of desensitization. In control cells, desensitization is promoted significantly by 0.3 ng/ml FSH. In the presence of PKAI during preincubation, significant desensitization appeared for FSH doses higher than 1 ng/ml (Fig. 2C). Moreover, PKAI pretreatment resulted in a significant decrease in the FSH-induced desensitization, its maximum observed at 5 ng/ml FSH being reduced from 65% to 50%. Preincubation with the peptidic inhibitor of PKC (1 μM) induced the same shift in the FSH doses able to desensitize (Fig. 2D), and the extent of desensitization was decreased from 60% to 40%.

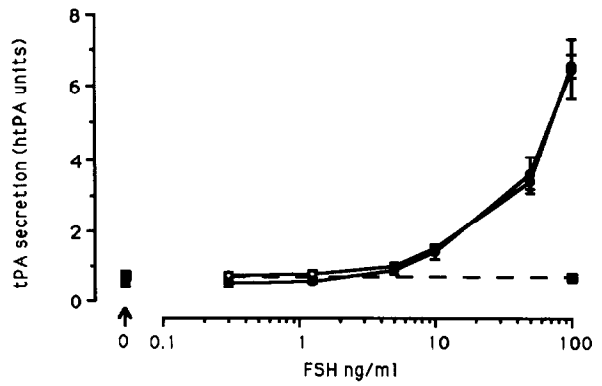

Fig. 3. Effect of cycloheximide pretreatment on FSH stimulated tPA secretion. Sertoli cells were preincubated with (●) or without (○) 5 μg/ml cycloheximide for 2.5 h. After washing, the cells were incubated for 4 h with increasing concentrations of FSH and tPA levels were determined. Control (■) consisted of the 4 h incubation with 5 μg/ml cycloheximide and 100 ng/ml FSH. Values are means ± S.E.M. of triplicate determinations of a representative experiment.

3.3. *Effect of protein synthesis inhibition on the desensitization of the tPA response*

Sertoli cells were exposed to 5 μg/ml cycloheximide for 10 min prior to the addition of desensitizating concentrations of the agents and during the 2.5 h preincubation. The cells were subsequently washed and challenged with 100 ng/ml FSH for 4 h, after which time secreted tPA in the media was measured. Cycloheximide was completely removed by the cell washing, as assessed by the full tPA response recovered by Sertoli cells to FSH stimulation (Fig. 3).

The perfect superimposition of the FSH stimulatory curves obtained in cells preincubated with or without 5 μg/ml cycloheximide shows that, the presence of cycloheximide alone during pretreatment did not modify the subsequent FSH-stimulated tPA production (Fig. 3). The same concentration of cycloheximide during the incubation period is, however, able to completely abolish tPA secretion induced by FSH 100 ng/ml, reflecting inhibition of tPA synthesis (Fig. 3).

In the desensitization experiments, cycloheximide did not significantly affect the Pdbu- (Fig. 4A) or db-cAMP- (Fig. 4B) induced desensitization of the tPA response to subsequent FSH stimuation. 10 and 100 nM Pdbu induced 40% and 50% inhibition, respectively, of the subsequent FSH-stimulated tPA secretion by Sertoli cells. Cycloheximide present before and during preincubation did not modify the extent of Pdbu-induced desensitization (Fig. 4A). Preincubation of the Sertoli cells with 10^{-5} M or 5×10^{-5} M dbcAMP promoted 25% and 40% inhibition, respectively, of the subsequent 100 ng/ml FSH-stimulated tPA secretion. Cycloheximide did not alter this dbcAMP-induced desensitization (Fig. 4B).

Cycloheximide affected the FSH-induced desensitization pattern of the tPA secretion subsequently stimulated by 100 ng/ml FSH (Fig. 5). Cycloheximide present during preincubation, without altering the appearance of desensitization promoted at the lowest FSH doses (< 1 ng/ml), significantly prevented the desensitization induced by higher FSH doses. A maximum of 40% of desensitization was observed with 0.6 ng/ml FSH in the presence of cycloheximide, whereas 70% of the desensitization could be achieved with 5 ng/ml FSH in control cells.

4. Discussion

We have previously described how low concentrations of FSH induced desensitization of Sertoli cells by measuring the secretion of tPA as an end point of this process [1]: it was not due to down-regulation of the receptors and did not affect the properties of the successive proteins involved in the transduction pathway. Rather, it is due to receptor G_s protein uncoupling. We then demonstrated an heterologous component of the Sertoli cell tPA response desensitization under subsequent FSH stimulation: it can be promoted either with low FSH concentrations (10^{-11} M), isoproterenol, AMPc analogues or a PKC activator. This led us to postulate that protein kinases were involved in this process [1].

To lend further support to the involvement of these different kinases in FSH-induced desensitization and to try to discriminate their respective roles, we used specific peptidic inhibitors

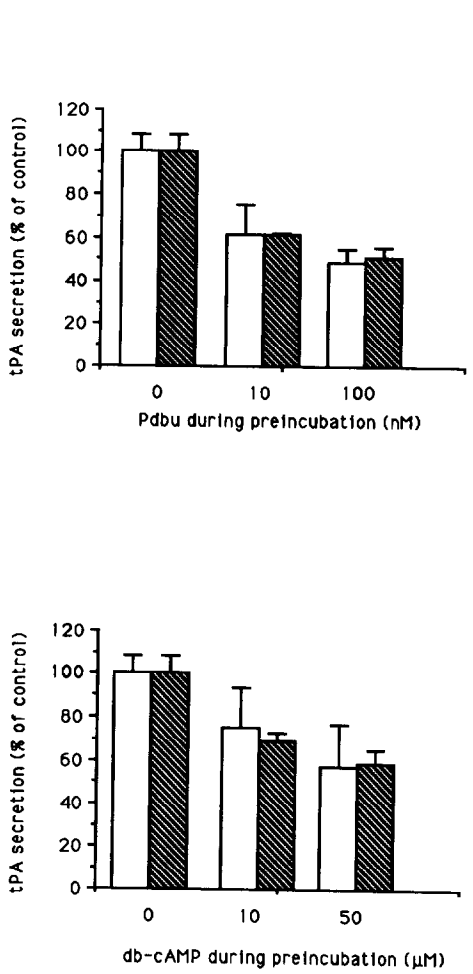

Fig. 4. Effect of cycloheximide on desensitization of tPA secretion induced by PKC or PKA activators. Sertoli cells were first incubated for 10 min with (hatched bars) or without (open bars) 5 μg/ml cycloheximide. Subsequently the indicated concentrations of Pdbu (A) or db-cAMP (B) were added and the preincubations continued for 2.5 h. After washing, the cells were incubated for 4 h with 100 ng/ml FSH. tPA levels were determined and expressed as a percentage of the mean tPA levels produced after stimulation by 100 ng/ml FSH by not desensitized Sertoli cells preincubated with or without cycloheximide. Values are means ± S.E.M. of triplicate determinations of a representative experiment.

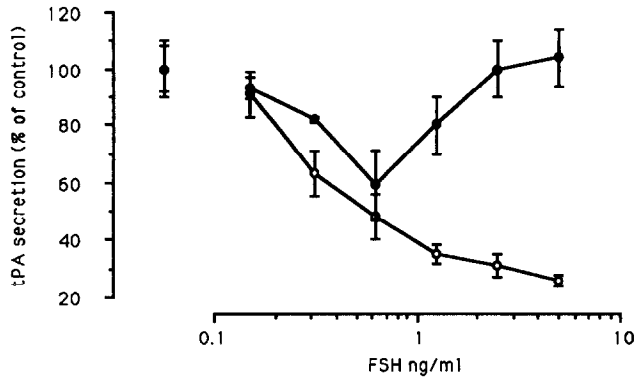

Fig. 5. Effect of cycloheximide on desensitization of tPA secretion induced by FSH. Sertoli cells were first incubated for 10 min with (●) or without (○) 5 μg/ml cycloheximide. Subsequently the indicated concentrations of FSH were added and the preincubations continued for 2.5 h. After washing, the cells were incubated for 4 h with 100 ng/ml FSH. tPA levels were determined and expressed as a percentage of the mean tPA levels produced after stimulation by 100 ng/ml FSH by not desensitized Sertoli cells preincubated with or without cycloheximide. Values are means ± S.E.M. of triplicate determinations of a representative experiment.

of PKA and PKC which interact with the substrate sites of the catalytic domains of these enzymes. At the concentration of 1 μM used, these protein kinase inhibitors are effective in completely blocking the activity of the kinases in a specific manner. Although protein kinase inhibitors prevent the onset of desensitization induced by 0.1–1 ng/ml FSH, they only partially (20%) inhibit desensitization induced by higher FSH concentrations. These peptidic inhibitors clearly identify a first part of the desensitization as being PKA- and PKC-mediated. These results can be linked to our previous studies on the effect of a pharmacological cell permeable kinase inhibitor H-9 (isoquinoline derivative) during the preincubation desensitizing period [1]. $5 \cdot 10^{-5}$ M H-9 partially prevented FSH-induced desensitization, the maximum of desensitization being reduced from 65 to 30%. At the concentration used, however, although H-9 mainly inhibits PKA it is also known to inhibit a number of other protein kinases, notably PKC.

Nevertheless, none of these inhibitors was effective in completely impeding FSH-induced desensitization. We can thus clearly distinguish an additional mechanism in the FSH-induced desensitization. This residual desensitization indicates that FSH regulates cell responsiveness by multiple mechanisms, one of them affected by inhibition of PKA and PKC and the other(s) not mediated by these protein kinases. This is in agreement with our previous result [1] showing that all the agents producing desensitization of the tPA response also stimulate the protein kinases but that this desensitization form is only a portion of FSH-induced desensitization. Indeed, an additional homologous component (FSH/FSH) is developed over the heterologous one.

Since maximum FSH desensitization required pretreatment of the cells for 2.5 h, the possibility that this inhibition required protein synthesis was examined by the use of cycloheximide. Heterologous desensitization (45% of inhibition) induced by PKC activation with Pdbu was not altered in the presence of cycloheximide during the preincubation period. This confirms that the PKC-mediated desensitization of the FSH-stimulated tPA response in Sertoli cells is independent of protein synthesis. Similarly, heterologous desensitization (40% of inhibition) induced by PKA activation with db-cAMP was unaffected by cycloheximide pretreatment. This allowed us to distinguish a PKA-mediated desensitization of the tPA response in Sertoli cells independent of protein synthesis.

Although cycloheximide does not affect desensitization induced by 0.1–1 ng/ml FSH, it abolished desensitization promoted by 1–5 ng/ml FSH (Fig. 5). This reflects the fact that there are two components in desensitization; one which is promoted by low FSH doses (0.1–1 ng/ml) and which is not dependent of on-going proteins synthesis, and a second one which is promoted by higher FSH doses (2.5 and 5 ng/ml) and which appears to be dependent on protein synthesis. Concerning the second component, an effect of cycloheximide on tPA mRNA translation and synthesis can be put forward. It must be noted that cycloheximide alone during preincubation has no effect on subsequent FSH-stimulated tPA production by Sertoli cells, as confirmed by Fig. 3. However, inhibition by cycloheximide of the desensitization occurs at FSH concentrations that are able to slightly increase cAMP levels close to the threshold triggering tPA production [10]. The possibility that these FSH concentrations, combined with cycloheximide during the preincubation, were able to increase the levels of tPA mRNA and could promote an additional tPA secretion during subsequent incubation, which would overlap the desensitization process studied, must be taken into consideration. A detailed functional analysis of this process at the level of the tPA mRNA is required to verify this hypothesis.

Another hypothesis is the stimulation of a cytosolic proteic factor synthesis by desensitizing FSH doses, which would subsequently interfere with the FSH action. Protein kinase inhibitors [13] or phosphodiesterases [14] are not likely candidates because the site of lesion of the desensitization observed is localized upstream of the production of cAMP [1]. Therefore, we suggest a direct involvement of this putative protein in the uncoupling of the receptor/G_s protein, similarly to the arrestin-like proteins.

The FSH receptor belongs to the wide superfamily of G protein-coupled receptors which exhibit such desensitization patterns, including of the more studied ones, the β-adrenergic, muscarinic, rhodopsine, cholecystokinine and luteotropin receptors [15–20]. Although little is known concerning the short-term desensitization of the FSH receptor, it is reasonable to assume that its activity is controlled in a similar way.

According to our results and those in the literature, the following working hypothesis can be suggested: FSH binds to its receptor under basal or slightly stimulating concentrations and induces the reduction in response of the Sertoli cells to its subsequent stimulation. This desensitization is characterized by an uncoupling of the FSH receptor and the G_s protein. Triggered also by other Sertoli cell stimulators, the heterologous form of desensitization would involve the activation of protein kinases and phosphorylation of specific proteins. We can thus suggest that the FSH receptor and/or G_s protein could be targets for these kinases. Although these studies reveal an uncoupling between the FSH receptor and its transduction pathway and strongly imply a role for PKA and PKC in causing the heterologous desensitization, further studies will be necessary to establish the concept that direct phosphorylation of the FSH receptor by these kinases does occur in Sertoli cells in response

to FSH or other stimulators and triggers desensitization. Recently, the phosphorylation of the FSH receptor by FSH and PKC and its negative impact on the receptor function have been demonstrated, but in a cell line stably transfected with the rat FSH receptor cDNA [21]. Moreover, the molecular basis of the homologous component of the FSH-induced desensitization and the involvement of protein synthesis also require further investigation.

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