Characterization of the homologous and heterologous desensitization of rat Leydig-tumour-cell adenylate cyclase

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(Received 16 January 1984/Accepted 7 March 1984)

The homologous and heterologous desensitization of rat Leydig-tumour-cell adenylate cyclase induced by lutropin (LH) was characterized with the aid of forskolin and cholera toxin. Forskolin stimulated cyclic AMP production in a dose-dependent manner, with linear kinetics up to 2 h. Forskolin also potentiated the action of LH on cyclic AMP production, but was only additive with cholera toxin. Preincubation of rat Leydig tumour cells with LH (1.0 $\mu\text{g/ml}$) for 1 h produced a desensitization of the subsequent LH (1.0 $\mu\text{g/ml}$)-stimulated cyclic AMP production, whereas the responses to cholera toxin (5.0 $\mu\text{g/ml}$), forskolin (100 μM), LH plus forskolin or cholera toxin plus forskolin were unaltered. In contrast, preincubation with LH for 20 h produced a desensitization to all the stimuli tested. When rat Leydig tumour cells were preincubated for 1 h with forskolin or dibutyryl cyclic AMP, the only subsequent response that was significantly altered was that to LH plus forskolin after preincubation with forskolin. However, preincubation for 20 h with forskolin or dibutyryl cyclic AMP induced a desensitization to all stimuli subsequently tested. LH produced a rapid (0–1 h) homologous desensitization, which was followed by a slower (2–8 h)-onset heterologous desensitization. Forskolin and dibutyryl cyclic AMP were only able to induce heterologous desensitization. The rate of desensitization induced by either forskolin or dibutyryl cyclic AMP was similar to the rate of heterologous desensitization induced by LH. These results demonstrate that in purified rat Leydig tumour cells LH produces an initial homologous desensitization of adenylate cyclase that involves a cyclic AMP-independent lesion at or proximal to the guanine nucleotide regulatory protein (G-protein). This is followed by heterologous desensitization, which can also be induced by forskolin or dibutyryl cyclic AMP, thus indicating that LH-induced heterologous desensitization of rat Leydig-tumour-cell adenylate cyclase involves a cyclic AMP-dependent lesion that is after the G-protein.

It is now well established that many hormones activate adenylate cyclase via a specific receptor and a G-protein (Rodbell, 1980) and that the same hormones can produce a subsequent desensitization of that adenylate cyclase system. Two types of desensitization of adenylate cyclase have been described, a specific or homologous desensitization and a general or heterologous desensitization (Su *et al.*, 1978). The mechanisms of both types of desensitization are still unclear; however, homologous desensitization may involve modifications to the hormone receptor or the coupling of the hormone receptor to G-protein (Iyengar *et al.*, 1981;

Abbreviations used: G-protein, guanine nucleotide regulatory protein; LH, lutropin.

Dix *et al.*, 1982), which is probably a cyclic AMP-independent process (Ezra & Salomon, 1980; Dix *et al.*, 1982), whereas heterologous desensitization may involve modification of the G-protein and may be cyclic AMP-mediated (Hunzicker-Dunn & Birnbaumer, 1981).

We have characterized a system *in vitro* in which LH induces a specific desensitization of Leydig-tumour-cell adenylate cyclase (Dix & Cooke, 1981). In this system LH produces a rapid (within 15–60 min) desensitization to subsequent stimulation with LH; however, the response to cholera toxin in intact cells and to guanine nucleotides and NaF in plasma membranes from LH-desensitized cells is unaltered (Dix *et al.*, 1982). In the same

studies it was shown that exposure of the cells to dibutyryl cyclic AMP had no effect on subsequent responses of intact cells or plasma membranes. During the initial (0–1 h) desensitization with LH the binding of ^{125}I -labelled human choriogonadotropin was also unaltered. These findings led to the conclusions that LH induces a lesion at or proximal to the G-protein and that this lesion is specific to the LH response and is probably not mediated by cyclic AMP (Dix *et al.*, 1982).

Since the original observation by Metzger & Lindner (1981) that forskolin, a diterpene isolated from the root of *Coleus forskohlii*, is a potent activator of adenylate cyclase, extensive studies have been carried out to elucidate the site of action of this compound. Seamon & Daly (1981) proposed that forskolin acts directly on the catalytic unit of adenylate cyclase, since it activates the soluble adenylate cyclase from rat testis and the cyclase of cyc⁻ S49 lymphoma-cell mutants, two systems supposedly devoid of a functional G-protein (Ross & Gilman, 1977; Seamon & Daly, 1981). Forskolin also activates adenylate cyclase from rabbit liver that was prepared free of G-protein (Ross, 1982). However, studies on forskolin-activated S49 lymphoma-cell mutants indicate some involvement of the G-protein (Darfler *et al.*, 1982), and the forskolin activation of adenylate cyclase in cerebral-cortex plasma membranes was potentiated by GTP. Also many studies have reported that forskolin was able to potentiate the actions of various hormones in a variety of cell types (Seamon & Daly, 1981; Fradkin *et al.*, 1982; Insel *et al.*, 1982). These conflicting reports on the mechanism of action of forskolin have subsequently been explained. Wong & Martin (1983) have shown, using a photoreactive GTP analogue, which abolishes the activation of adenylate cyclase via the G-protein, that forskolin can act via a G-protein and also independently of this protein.

In order to characterize further the desensitization of rat Leydig-tumour-cell adenylate cyclase, the present study has compared the stimulatory and desensitizing effects of forskolin on cyclic AMP production with those of LH. These results clearly show that, whereas LH produces both a rapid homologous desensitization and a slower-onset heterologous desensitization, forskolin only produces the slow-onset heterologous desensitization. This heterologous desensitization can also be induced by dibutyryl cyclic AMP.

Materials and methods

Sheep LH (NIH S20; 1.9 i.u. NIH SI/mg) was a gift from the Endocrinology Study Section, National Institutes of Health, Bethesda, MD, U.S.A. Methylcellulose, Hepes [4-(2-hydroxyethyl)-1-

piperazine-ethanesulphonic acid], cholera toxin and bovine serum albumin (fraction V) were purchased from Sigma Chemical Co. Forskolin (7 β -acetoxy-8,13-epoxy-1 α ,6 β ,9 α -trihydroxylabda-11,14-dione; M_r 410) was purchased from Calbiochem. Percoll was purchased from Pharmacia Fine Chemicals, and 3-isobutyl-1-methylxanthine was purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Na ^{125}I (carrier-free) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Dulbecco's modified Eagle's minimum essential medium, without NaHCO₃, was purchased from Gibco.

Preparation of Leydig tumour cells

The Leydig tumour used for these studies has been previously characterized (Cooke *et al.*, 1979). The Leydig tumour was grown in Fischer rats, and the cells were isolated and purified by using Percoll density gradients, as previously described (Dix *et al.*, 1982). Cells with a density of 1.070 g/ml were used in all studies.

Cell incubations

The purified Leydig cells were suspended in suspension medium [Dulbecco's modified Eagle's minimum essential medium: 0.1% (w/v) bovine serum albumin, 10 mM-Hepes and 0.1% (w/v) methylcellulose (pH 7.4)]. In preincubation experiments, cells were incubated with either suspension medium alone or suspension medium containing LH (1 $\mu\text{g/ml}$), forskolin (100 μM) or dibutyryl cyclic AMP (1 mM) in a shaking water bath at 32°C. After preincubation, the cells were washed three times by centrifugation (200 g for 10 min) and then resuspended in fresh media. Except where indicated, the incubation medium contained isobutylmethylxanthine (0.5 mM). Incubations were carried out in the absence and presence of LH (1 $\mu\text{g/ml}$), forskolin (1–100 μM), LH plus forskolin, cholera toxin (5 $\mu\text{g/ml}$) or cholera toxin plus forskolin, for various times. At the end of the incubation period cyclic AMP was extracted from the cells by addition of HClO₄ (final concn. 0.5 M) and then neutralized with K₃PO₄ (final concn. 0.24 M). Cyclic AMP was then determined by radioimmunoassay by the method of Steiner *et al.* (1972), modified by the acetylation procedure described by Harper & Brooker (1975).

Results

In isolated rat Leydig tumour cells, forskolin stimulated cyclic AMP production in a dose-dependent manner. As shown in Fig. 1, concentrations of forskolin up to 100 μM did not produce a maximal response. However, forskolin was not soluble in the medium at higher concentrations,

Fig. 1. Effect of forskolin concentration on basal and LH-stimulated cyclic AMP production

Leydig tumour cells prepared as described in the Materials and methods section were incubated alone (B; □), with different concentrations of forskolin in the absence (○) or presence (●) of LH (1.0 μg/ml), or with LH alone (■) for 1 h at 32°C. All incubations contained isobutylmethylxanthine (0.5 mM). The cyclic AMP was determined as described in the Materials and methods section. Results are means ± S.D. (represented by the bars) for duplicate determination on triplicate samples.

and therefore further studies to determine the maximum response could not be carried out. The amount of cyclic AMP produced in 1 h by forskolin (100 μM) is greater than that produced by a maximal stimulating concentration of LH (1.0 μg/ml) (Dix *et al.*, 1982) (Fig. 1). At all concentrations of forskolin tested, a marked potentiation was seen when LH was also present in the incubations (Fig. 1).

In agreement with previous results, the rate of cyclic AMP production stimulated by LH was non-linear (Fig. 2), and this has been shown to be due to desensitization (Dix *et al.*, 1982). The kinetics of cyclic AMP production stimulated by forskolin were linear up to 2 h. In contrast, the kinetics of the forskolin + LH-stimulated cyclic AMP production showed the characteristics of the kinetics of these

Fig. 2. Effect of forskolin, LH or forskolin + LH on the kinetics of cyclic AMP production

Leydig tumour cells prepared as described in the Materials and methods section were incubated at 32°C in media containing isobutylmethylxanthine (0.5 mM) for various times in the presence of LH (○; 1 μg/ml), forskolin (□; 100 μM), LH (1 μg/ml) + forskolin (■; 100 μM) or in medium alone (●), and the cyclic AMP contents were determined as described in the Materials and methods section. Results are means ± S.D. (represented by the bars) for duplicate determinations on triplicate samples.

two stimulants alone (Fig. 2a). Although the initial rate of cyclic AMP production was faster than that with LH or forskolin alone, once the LH-stimulated component was desensitized the rate of production was similar to that with forskolin alone (Fig. 2).

In contrast with the two-component kinetics seen with LH + forskolin-stimulated cyclic AMP production, a maximal concentration of cholera toxin (5 μg/ml) and forskolin (100 μM) produced linear kinetics (Fig. 3). After an initial characteristic lag phase, the kinetics of cholera-toxin-stimulated cyclic AMP production were linear for up to 2 h (Fig. 3). As seen in Fig. 1, the kinetics of for-

Fig. 3. Effect of forskolin, cholera toxin or forskolin + cholera toxin on the kinetics of cyclic AMP production

Leydig tumour cells prepared as described in the Materials and methods section were incubated at 32°C in media containing isobutylmethylxanthine (0.5 mM) for various times in the presence of cholera toxin (○; 5 µg/ml), forskolin (□; 100 µM), cholera toxin (5 µg/ml) + forskolin (■; 100 µM) or with medium alone (●). The cyclic AMP content was then determined as described in the Materials and methods section. Results are means ± S.D. (represented by the bars) for duplicate determinations on triplicate samples.

skolin-stimulated cyclic AMP production were also linear. The combination of forskolin and cholera toxin also stimulated cyclic AMP production with linear kinetics. Whereas the initial rate of cyclic AMP production (0–15 min) stimulated by LH + forskolin showed a clear potentiation at all time points studied (Fig. 2), the cholera-toxin + forskolin-stimulated cyclic AMP production only showed additive effects (Fig. 3).

Preincubation of rat Leydig tumour cells with LH (1.0 µg/ml) for 1 h produced a desensitization of the subsequent LH-stimulated cyclic AMP production, whereas the responses to cholera toxin, forskolin, LH + forskolin or cholera toxin + forskolin were unaltered (Table 1). In contrast, preincubation of the cells with LH for 20 h resulted in a desensitization of all subsequent responses (Table 1). Preincubation of rat Leydig tumour cells for 1 h with forskolin (100 µM) or dibutyryl cyclic AMP (1 mM) had no effect on the subsequent responses to

Table 1. Effect of pretreatment with LH, forskolin or dibutyryl cyclic AMP for 1 h or 20 h on the subsequent cyclic AMP production
Leydig tumour cells prepared as described in the Materials and methods section were incubated at 32°C for 20 h, either alone or with LH (1 µg/ml), forskolin (100 µM) or dibutyryl cyclic AMP (1 mM), or for 19 h with medium alone and for the final 1 h with these agents. The cells were then washed (three times) and incubated for a further 1 h at 32°C in either medium containing isobutylmethylxanthine (0.5 mM) or the same medium in the presence of LH (1.0 µg/ml), cholera toxin (5.0 µg/ml), forskolin (100 µM), LH + forskolin or cholera toxin + forskolin. The cyclic AMP was then determined as described in the Materials and methods section. Results are means ± S.D. for duplicate determinations on triplicate samples. Values for incubation with medium alone were compared with those for each preincubation condition by Student's *t* test: **P* < 0.05.

Preincubation conditions	Time (h)	Cyclic AMP production (pmol/h per 10 ⁶ cells)					
		Basal	LH	Cholera toxin	Forskolin	LH + forskolin	Cholera toxin + forskolin
Medium	20	2.1 ± 0.5	23.7 ± 1.8	97.9 ± 3.6	120.3 ± 3.8	291.1 ± 9.4	266.3 ± 27.8
	1	1.7 ± 0.1	1.84 ± 0.2*	90.1 ± 4.8	132.2 ± 2.2*	270.4 ± 8.2	248.1 ± 11.7
LH	20	1.9 ± 0.1	1.4 ± 0.1*	55.5 ± 1.5*	44.7 ± 1.5*	70.5 ± 2.8*	101.5 ± 2.9*
	1	1.8 ± 0.1	27.5 ± 0.6	94.9 ± 6.9	119.1 ± 3.3	209.8 ± 9.8*	231.9 ± 10.7
Forskolin	20	1.1 ± 0.1*	3.5 ± 0.1*	12.7 ± 0.8*	7.3 ± 0.3*	26.8 ± 0.7*	30.3 ± 0.7*
	1	4.5 ± 1.1*	29.7 ± 2.4	86.1 ± 8.4	110.0 ± 10.1	284.7 ± 10.9	270.1 ± 3.8
Dibutyryl cyclic AMP	20	5.1 ± 1.9*	5.4 ± 0.8*	13.1 ± 2.7*	35.7 ± 4.1*	95.1 ± 5.9*	101.1 ± 4.6*

Fig. 4. Effect of time of pretreatment with LH or forskolin on the subsequent LH-, forskolin- or LH + forskolin-stimulated cyclic AMP production

Leydig tumour cells prepared as described in the Materials and methods section were preincubated at 32°C for various times with (a) LH (1.0 µg/ml) or (b) forskolin (100 µM), washed three times and incubated for 1 h at 32°C in media containing isobutylmethylxanthine (0.5 mM) in the presence of LH (●; 1.0 µg/ml), forskolin (△; 100 µM) or LH (1.0 µg/ml) + forskolin (□; 100 µM). The cyclic AMP content was then determined as described in the Materials and methods section. Results, expressed as percentage of control values, are means ± s.d. (represented by the bars) for duplicate determinations on triplicate samples.

LH, cholera toxin, forskolin or cholera toxin + forskolin, but the response to LH + forskolin was decreased by forskolin preincubation (Table 1). However, this decrease was not as dramatic as that when the cells were incubated for 20 h with forskolin or dibutyryl cyclic AMP, which caused a significant decrease in all subsequent responses (Table 1).

In order to investigate further the difference in time of desensitization, cells were incubated for up to 8 h with either LH (1.0 µg/ml) or forskolin (100 µM), and the subsequent responses to various stimuli were examined. Consistent with the results in Table 1, LH produced a rapid desensitization to a second challenge with LH, the cells showing complete desensitization by 2 h (Fig. 4a). However, the rate of desensitization of the forskolin response was slower and only became sig-

nificantly different from the control response by 4 h, and reached the degree of desensitization seen with LH by 8 h (Fig. 4a). The pattern of response to LH + forskolin in these LH-desensitized cells was a combination of the patterns with LH and with forskolin alone, with an initial rapid rate of desensitization followed by a slower decline to a degree of desensitization similar to that of the response to either LH or forskolin by 8 h (Fig. 4a).

In contrast with the different rates of LH-induced desensitization, forskolin-induced desensitization was similar for all three stimuli investigated, with only the slower rate of desensitization being apparent (Fig. 4b).

In order to study the differences between the specific desensitization induced by LH and the more general desensitization produced by forskolin or dibutyryl cyclic AMP, it was considered advantageous to find a second hormone or neurotransmitter which stimulated cyclic AMP production in rat Leydig tumour cells. Cells were incubated either alone or in the presence of forskolin (100 µM) with histamine, adenosine, prostaglandins E₂, F₂, E₁ and I₂, 5-hydroxytryptamine, adrenaline and carbachol, at 0.1 and 10 µM, and with glucagon and prolactin at 1 and 10 µg/ml for 1 h at 32°C in the presence of isobutylmethylxanthine (0.5 mM). The cyclic AMP productions were then determined. Under these conditions none of the compounds tested produced a significant effect on basal or forskolin-stimulated cyclic AMP production. During the course of these studies prostaglandin E₁, isoprenaline and LH were incubated with a Leydig-tumour-cell-suspension that had not undergone previous purification on Percoll density gradients, and the cyclic AMP productions were compared with those of purified cells. As shown in Table 2, although isoprenaline, prostaglandin E₁ and LH stimulated cyclic AMP production significantly in the crude cell preparation, only LH stimulated cyclic AMP production in the purified rat Leydig tumour cells.

Discussion

Many hormones have been reported to cause desensitization of adenylate cyclase. However, no single mechanism has been demonstrated, and the time of onset, the degree and the hormone-specificity of desensitization vary according to the hormone and cell type being studied. In the present investigation we have demonstrated the need to obtain a single population of cells, since in the crude Leydig-tumour-cell preparation LH, isoprenaline and prostaglandin E₁ stimulated cyclic AMP production, but only LH stimulated cyclic AMP production in cells that had been purified. The importance of hormone exposure time is also demonstrated, since exposure of rat Leydig tumour

Table 2. Effect of purification of rat Leydig tumour cells on Percoll density gradients on isoprenaline-, prostaglandin E₁- and LH-stimulated cyclic AMP production

Leydig tumour cells were prepared as described in the Materials and methods section and incubated as a cell suspension either before or after purification on a Percoll gradient in media containing isobutylmethylxanthine (0.5 mM) at 32°C for 1 h in the presence of isoprenaline (100 µM), prostaglandin E₁ (100 µM) or LH (10 µg/ml). The cyclic AMP content was then determined as described in the Materials and methods section. Results are means $\pm$ D. for duplicate determinations on triplicate samples. Hormone-stimulated values were compared with basal values by Student's *t* test: **P* < 0.05.

Rat Leydig tumour cells	Cyclic AMP Production (pmol/h per 10 ⁶ cells)			
	Basal	Isoprenaline (100 µM)	Prostaglandin E ₁ (100 µM)	LH (1 µg/ml)
Crude	11.3 $\pm$ 1.7	37.6 $\pm$ 9.3*	121.3 $\pm$ 16.7*	153.3 $\pm$ 8.6*
Purified	13.7 $\pm$ 4.2	20.0 $\pm$ 4.5	21.5 $\pm$ 6.3	325.1 $\pm$ 14.4*

cells to LH for 1–2 h produced only a LH-specific desensitization which had no effect on either cholera-toxin- or forskolin-stimulated cyclic AMP production, whereas a longer exposure time (8–20 h) produced a general desensitization to LH, cholera toxin and forskolin. In order to characterize fully a hormone-induced desensitization, it is advantageous to have a cell type that has more than one hormone that activates adenylate cyclase. However, in the rat Leydig-tumour cells, no hormone other than LH has yet been found to activate adenylate cyclase. It is possible therefore that the initial homologous or hormone-specific desensitization referred to in the present study may be heterologous with respect to other hormones.

In the present study the use of forskolin and cholera toxin as secondary stimulants and the use of forskolin as a desensitizing agent have enabled a characterization of both homologous and heterologous desensitization in rat Leydig tumour cells. Forskolin has been reported to stimulate cyclic AMP production in a variety of cell types (Seamon & Daly, 1981; Siegl *et al.*, 1982). In common with these findings, forskolin stimulated cyclic AMP production in a dose-dependent manner in isolated rat Leydig tumour cells. The stimulation of cyclic AMP production by forskolin was rapid and did not have a lag phase, as reported by Clark *et al.* (1982) for S49 lymphoma cells. The kinetics of forskolin-stimulated cyclic AMP production were linear for at least 2 h, which is in sharp contrast with the kinetics of LH-stimulated cyclic AMP production, which indicated a decline in the rate of cyclic AMP production within 15 min of exposure to LH. Forskolin acted synergistically with LH, but only had an additive effect on cholera-toxin-stimulated cyclic AMP production. This is again in common with the findings of other studies (Fradkin *et al.*, 1982; Forte, 1983; van Sande *et al.*, 1983). It is apparent from the kinetics of LH + forskolin-stimulated cyclic AMP production that the synergism is lost once the LH response is desensitized.

Since we have previously shown that LH-induced desensitization involves a lesion between the LH receptor and the G-protein (Dix *et al.*, 1982), the kinetics of LH + forskolin-stimulated cyclic AMP production suggests that the coupling between the LH receptor and the G-protein needs to be functional for synergism between LH and forskolin. Darfler *et al.* (1982) came to similar conclusions for the actions of isoprenaline and forskolin on genetic variants of S49 lymphoma cells.

It appears that LH-induced desensitization involves at least two independent mechanisms; the first, as previously characterized (Dix *et al.*, 1982), involves a lesion between the LH receptor and the G-protein. This lesion is an early event in desensitization and is hormone-specific. Such a mechanism has also been described for glucagon in hepatocytes (Heyworth & Houslay, 1983) and for β -adrenoceptor agonists in many cell types (Su *et al.*, 1980; Pike & Lefkowitz, 1980; Green & Clark, 1981; Fishman *et al.*, 1981). This early lesion may involve a cyclic AMP-independent phosphorylation of either the receptor or the G-protein (Hunzicker-Dunn *et al.*, 1979). There is some evidence that LH-induced desensitization may involve a GTP-dependent phosphorylation (Ezra & Salomon, 1980; Levi *et al.*, 1982). We have previously demonstrated that the functional transfer of G-protein from human erythrocytes can reverse LH-induced desensitization in rat tumour Leydig cell plasma membranes (Dix & Cooke, 1982) indicating that the G-protein is the site of the early lesion in rat Leydig tumour cells. It may therefore be possible that the G-protein is able to undergo a GTP-dependent autophosphorylation which uncouples it from the hormone receptor. Once this initial desensitization has occurred, continued presence of hormone produces a further alteration in the adenylate cyclase system, which causes a general desensitization. Since this heterologous desensitization can be induced by forskolin and dibutyl cyclic AMP, it would appear to be cyclic

AMP-dependent. Wong & Martin (1983) have reported that forskolin has at least some of its action distal to the G-protein. Forskolin is unable to stimulate cyclic AMP production in rat Leydig tumour cells that has been desensitized with LH, dibutyryl cyclic AMP or forskolin for 20 h; it therefore appears that the lesion produced in heterologous desensitization is due to an alteration of the catalytic unit. However, Brooker *et al.* (1983) have reported that inhibitors of protein synthesis can decrease forskolin-stimulated cyclic AMP production while having little effect on hormone-stimulated cyclic AMP production, and suggest that the action of forskolin is mediated via an intermediary protein which is a previously unrecognized component of the adenylate cyclase system. If this were the case, heterologous desensitization may involve alteration of such a protein.

In conclusion, we have demonstrated that in purified rat Leydig tumour cells LH produces an initial homologous desensitization of adenylate cyclase that involves a lesion at or proximal to the G-protein and is independent of cyclic AMP. This is followed by heterologous desensitization, which can also be induced by forskolin or dibutyryl cyclic AMP, thus indicating that heterologous desensitization of rat Leydig-tumour-cell adenylate cyclase is cyclic AMP-dependent and involves a lesion distal to the G-protein. It is also stressed that to study these desensitization phenomena a purified cell system is essential.

We are grateful to the Medical Research Council for financial support, to the National Pituitary Agency, Bethesda, MD, U.S.A., for the gift of sheep LH, and to Miss B. E. Macey for typing the manuscript.

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