Control of photoperiodic inhibition of luteinizing hormone secretion by dopaminergic and serotonergic systems in ovariectomized Ile-de-France ewes supplemented with oestradiol

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The role of dopaminergic and serotonergic systems on LH secretion was investigated in Ile-de-France ewes under different artificial inhibitory photoperiodic regimens. All animals were ovariectomized at the end of the breeding season, chronically treated with an oestradiol implant, and subjected to various changes in daylength for 9 months to inhibit or stimulate their LH secretion. Plasma LH concentration was assessed by taking blood samples twice a week throughout the experiment. The effects of acute intravenous injections of the dopaminergic₂ receptor antagonist pimozide (0.08 mg kg^{-1}) and the 5-hydroxytryptamine₂ (5HT₂) receptor antagonist cyproheptadine (3 mg kg^{-1}) on LH pulsatility were assessed during challenges in four different situations: (1) long days (LD); (2) before short-day response (SD); (3) during refractoriness to short days (RSD); and (4) during inhibition by long days (ILD). LH in blood samples collected twice a week remained low during long days (0.59 ± 0.03 ; mean $\pm$ SEM), increased 45 ± 1.5 days after the onset of short days and decreased 132 ± 4.9 days later when ewes became refractory to short days, whereas ewes subjected to long days after 91 short days stopped their neuroendocrine activity 19 days earlier (113 ± 4.7) ($P < 0.01$). In comparison with the pre-injection period, pimozide significantly increased the mean number of pulses in SD and RSD ewes, but not in LD and ILD ewes: SD: 0 versus 0.45 pulses in 4 h ($P < 0.02$); RSD: 0 versus 0.9 ($P = 0.05$). Cyproheptadine significantly increased the mean number of pulses in SD and RSD ewes: SD: 0 versus 1 ($P < 0.008$); RSD: 0 versus 1.5 ($P = 0.03$). An effect of cyproheptadine was shown in LD ewes (0 versus 0.5 ($P < 0.03$)), but it was less marked than in the same ewes under short-day photoperiod (SD ewes; $P < 0.05$). In ILD ewes, a small increase was observed (0 versus 0.33 ($P = 0.05$)) but it was less than in RSD ewes ($P < 0.03$). These results support the hypothesis of an inhibitory role of dopaminergic and serotonergic systems on LH pulsatile release and suggest that refractoriness to short days is due to activation of these two systems.

Introduction

In sheep under natural photoperiod, daylength variation induces changes in gonadotrophin secretion that are responsible for seasonal activity of reproductive function (Goodman *et al.*, 1981). Several studies have shown the role of two neural systems responsible for LH pulse frequency inhibition during natural anoestrus. The first system is oestradiol sensitive (Legan *et al.*, 1977) and could be mediated by catecholaminergic neurones since dopaminergic and adrenergic receptor antagonists increased LH pulsatility in intact but not in ovariectomized ewes (Meyer and Goodman, 1985; 1986). The second system involves a direct steroid-independent influence on tonic LH release (Goodman *et al.*, 1982) and could be controlled by serotonergic neurones, as 5-hydroxytryptamine₂ (5HT₂) receptor antagonists increase LH pulse frequency in ovariectomized

ewes not treated with oestradiol (Meyer and Goodman, 1986; Whisnant and Goodman, 1990). However, the relative part of each of these systems in the seasonal inhibition of gonadotrophin activity remains unknown.

Under artificial photoperiod, transfer of ewes from a long to a short photoperiod produces an increase in neuroendocrine sexual activity after about 50 days. However, prolonged exposure to short days causes a decrease in LH pulsatility after about 140 days, making the animals photorefractory. This photorefractory state is thought to be responsible for the termination of the breeding season in natural conditions (Robinson and Karsch, 1984). The neural mechanisms responsible for the transduction of photoperiodic signals into an endocrine response have not been completely elucidated. It is now established that melatonin mediates the reproductive response to both inductive and inhibitory daylengths (Bittman *et al.*, 1983; Bittman and Karsch, 1984) and that photorefractoriness is attributed to a disruption in the post-pineal processing of the photoperiodic message rather than to a change in the melatonin signal (Malpoux *et al.*, 1987).

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We therefore investigated the role of dopaminergic and serotonergic systems in different situations when the neuroendocrine LH activity was inhibited, in ovariectomized Ile-de-France ewes bearing subcutaneous (s.c.) oestradiol implants and maintained under artificial photoperiodic regimens. The effects of intravenous injections of antagonists to dopaminergic₂ and 5HT₂ receptors (pimozide and cyproheptadine, respectively) on LH pulsatility were assessed in four different situations: (1) in long days (LD); (2) before short days response (SD); (3) during short days refractoriness (RSD) and (4) during inhibition by long days (ILD).

Materials and Methods

Animals and photoperiodic treatments

Thirty-two Ile-de-France ewes, 2–9 years old, maintained outdoors were selected at random in December 1989. They were ovariectomized (3% halothane in oxygen for anaesthesia) in January at the end of the breeding season and simultaneously implanted s.c. with a 1.5 cm Silastic implant (internal diameter = 3.3 mm and external diameter = 4.6 mm) (Karsch *et al.*, 1973) containing crystalline oestradiol (Sigma Chemical Co., Strasbourg). Ewes were allocated according to weight and age and divided into two groups of equal numbers and from 2 February housed in a light-proof building under artificial lighting (300 lux at animal eye level) and subjected to two photoperiodic treatments (see Fig. 1a). All ewes were subjected to 90 days of long days (LD 16 h light:8 h dark; 2 February to 2 May) followed by 91 days of short days (SD 8 h light:16 h dark; 2 May to 2 August). From 2 August, 16 ewes were maintained under short days (refractory to short days; group RSD) and 16 ewes were again subjected to long days (inhibited by long days; group ILD) until the end of the experiment (16 October 1990).

Long-term LH neuroendocrine activity

The effect of photoperiodic treatments on neuroendocrine LH activity was assessed by monitoring long-term LH variations by collecting blood samples twice a week by venepuncture. Samples were immediately centrifuged for 30 min (5000 g) and plasma stored at -20°C until assay.

The time of onset and cessation of LH secretion were determined arbitrarily in each ewe when the concentrations of at least two consecutive LH samples were higher or below 0.8 ng ml^{-1} , respectively.

In each photoperiodic situation, drug treatments were given when the concentration of LH in serum of each ewe was below 0.8 ng ml^{-1} (three ewes were consequently excluded in each drug treatment).

Drugs

Antagonists to dopaminergic₂ receptors (pimozide) and to serotonergic₂ receptors (cyproheptadine) were used. Pimozide (Sigma Chemical Co., Strasbourg) was dissolved in 0.1 mmol tartaric acid l^{-1} at a concentration of 1.7 mg ml^{-1} and given intravenously at a dose of 0.08 mg kg^{-1} body weight. Cyproheptadine hydrochloride sesquihydrate (Aldrich Chemical Co, St Quentin-Fallavier) was dissolved in an equal volume of

ethanol–propanediol solution at a concentration of 35 mg ml^{-1} . Drugs were given intravenously at a dose of 3 mg kg^{-1} body weight. Each drug was dissolved less than 20 h before intravenous injection and stored at 4°C until use.

Drug treatments

Effect of pimozide and cyproheptadine on LH release in ewes in long days and before short-day response. Ewes received either pimozide ($n = 11$) or cyproheptadine ($n = 10$) in long days (LD: 19 April) and the whole procedure was repeated in the same ewes before short-day response (SD: 7 June). Control ewes received either tartaric acid (vehicle 1: $n = 4$) or ethanol–propanediol (vehicle 2: $n = 4$).

Effect of pimozide and cyproheptadine on LH release in ewes refractory to short days or inhibited by long days. Ewes refractory to short days or inhibited by long days received either pimozide (RSD: $n = 4$ and ILD: $n = 6$) or cyproheptadine (RSD: $n = 5$ and ILD: $n = 6$). Two challenges were made (on 12 September and 16 October). Control ewes received either tartaric acid (vehicle 1) (RSD: $n = 2$ and ILD: $n = 2$) or ethanol–propanediol (vehicle 2) (RSD: $n = 2$ and ILD: $n = 2$).

During all challenges, blood samples were collected at 20 min intervals 4 h before and 4 h after intravenous injection of drugs or appropriate vehicles.

Hormone assays

LH was measured in a double-antibody radioimmunoassay (Pelletier *et al.*, 1982) modified by Montgomery *et al.* (1985). The sensitivity of the assay was 0.1 ng ml^{-1} and the intra- and interassay coefficients of variation were 7.0 and 9.8%, respectively. All samples from the same challenge were measured in the same assay.

LH pulse identification

LH pulses were analysed with the algorithm 'Monroe' (Merriam and Watcher, 1982). The G parameters (the number of standard deviations by which a peak must exceed the baseline in order to be accepted) were 3.98, 2.4, 1.68, 1.24 and 0.93 for G1–G5, respectively, for LH pulses requirements including 1 to 5 samples exceeding the baseline. The Baxter parameters describing the parabolic relationship between the hormone concentration of and the standard deviation (assay variation) of the concentration were 0.10520 (b_1 , the y intercept) 0.02516 (b_2 , the x coefficient) and 0.00039 (b_3 , the x^2 coefficient).

Statistical analysis

The effects of photoperiodic treatments on weekly samples of LH were compared using the one factor analysis of variance ANOVA (photoperiodic treatments: groups RSD or ILD) with time as a repeated measure (number of LH samples).

In drug treatments, the difference between mean LH concentration after and before treatment was calculated for each ewe. The same calculation was done for the number of LH pulses. These two variables were used for statistical analysis.

Results of mean LH concentration were analysed using ANOVA (SUPERANOVA, Abacus Concepts, Berkeley, CA)

Fig. 1. Experimental schedule (a): thirty-two Ile-de-France ewes were subjected to three months of long days (LD 16 h light:8 h dark) from 2 February to 2 May followed by three months of short days (SD 8 h light:16 h dark) until 2 August. Afterwards, until 16 October, 16 ewes were maintained under short days (RSD) and 16 ewes were subjected again to long days (ILD). (b) Effect of photoperiodic treatments on mean ($\pm$ SEM) long-term plasma luteinizing hormone (LH) concentration in ovariectomized (plus oestradiol treated) Ile-de-France ewes refractory to short days (RSD; $n = 13$ $\square$) and inhibited by long days (ILD; $n = 16$ $\blacksquare$). The arrows indicate dates of challenges with amine receptor antagonists or vehicles.

either with two factors: treatment (pimozide, cyproheptadine or control) and photoperiod (LD or SD as a repeated measure) or with 3 factors: treatment (pimozide, cyproheptadine or control), photoperiod (RSD versus ILD) and time as repeated measures. After ANOVA, effect of treatments on mean LH concentration were analysed using a paired *t* test (pre- versus post-injection periods) and effects of photoperiod were analysed using either paired *t* test (LD versus SD) or an unpaired *t* test (RSD versus ILD).

Effects of drug treatments on the number of LH pulses were analysed using the Wilcoxon test (pre- versus post-injection periods) and the Mann-Whitney *U* test (pimozide versus control and cyproheptadine versus control). Effects of photoperiod were analysed using the Wilcoxon test (LD versus SD) and the Mann-Whitney *U* test (RSD versus ILD) (Stat View, Abacus Concepts, Berkeley, CA).

Results

Effect of photoperiodic treatments on long-term LH secretion

From 2 February until 2 August, the LH secretory patterns were similar in groups RSD and ILD (Fig. 1b): the time of increase of LH concentrations was day 45.0 ± 1.5 (mean $\pm$ SEM)

in both groups (day 0 = 2 May). After 2 August, ANOVA revealed a significant effect of photoperiod on the reproductive response ($P < 0.01$): the decline of LH concentrations occurred earlier in group ILD (day 113.0 ± 4.7) than in group RSD (day 132.0 ± 4.9).

Effect of pimozide and cyproheptadine on LH release in ewes in long days and before short-day response

As there was no effect of vehicle treatments on the mean number of pulses or on mean LH concentration, whatever the photoperiodic situation, the hormonal results of control ewes were pooled (Figs 2 and 3).

ANOVA revealed a significant effect of drug treatments on mean LH concentration ($P < 0.004$). Groups pimozide and cyproheptadine significantly differed from the vehicle group for this parameter ($P < 0.04$ and $P = 0.01$, respectively).

After pimozide injection, no change was detected in LD ewes, whereas the mean number of pulses and mean LH concentration increased in SD ewes (0 versus 0.45 pulses in 4 h ($P < 0.02$) and 0.51 versus 0.66 ng ml⁻¹ ($P < 0.05$), respectively). The mean number of pulses in SD ewes differed from those of LD and control ewes ($P < 0.02$).

Fig. 2. Effect of cyproheptadine, pimoziide and vehicles on (a and c) the mean of number of pulses in 4 h ($\pm$ SEM) before ($\square$) and after ($\blacksquare$) injections in ovariectomized (plus oestradiol treated) ewes (a) in long days and (c) in the same ewes in short days and on (b and d) the mean plasma luteinizing hormone (LH) concentration during 4-h sampling period ($\pm$ SEM) before ($\blacksquare$) and after ($\square$) injection in ewes in (b) long days and in (d) short days. **, *** Significantly different from the pre-injection period ($P < 0.05$ and $P < 0.01$, respectively).

Fig. 3. Plasma luteinizing hormone (LH) concentrations during challenges in individual ewes receiving (a) cyproheptadine or (b) pimoziide or (c) vehicles in long days (LD; upper graph) and short days (SD; lower graph). (●) represents peaks of LH pulses. Time of injection of drugs or vehicles is indicated by the arrow.

After cyproheptadine injection, the mean number of pulses increased in LD ewes (0 versus 0.5 pulses in 4 h; $P < 0.03$) and in SD ewes (0 versus 1; $P < 0.008$). The mean LH concentration also rose: LD: 0.33 versus 0.62 ng ml⁻¹ ($P < 0.05$) and SD: 0.49

versus 0.87 ($P < 0.01$). The mean number of pulses was significantly higher in short than in long days ($P < 0.05$) and differed from control ewes (LD versus control ($P < 0.03$) and SD versus control ($P = 0.002$)).

Fig. 4. Effect of cyproheptadine, pimozide and vehicles on (a and c) the mean of number of LH pulses in 4 h ($\pm$ SEM) before ($\blacksquare$) and after ($\hatched$) injections in ovariectomized (plus oestradiol treated) ewes (a) inhibited by long days and (c) refractory to short days and (b and d) the mean plasma LH concentration during a 4-h sampling period ($\pm$ SEM) before ($\blacksquare$) and after ($\square$) injection in ewes (b) inhibited by long days and (d) refractory to short days. *, **, *** significantly different from the pre-injection period ($P = 0.05$, $P < 0.05$ and $P < 0.01$, respectively).

Effect of pimozide and cyproheptadine on LH release in ewes refractory to short days or inhibited by long days

As there was no effect of vehicle treatment on LH pulsatility or on mean LH concentration, results from control ewes were pooled in each photoperiodic group (Fig. 4).

ANOVA revealed an effect of photoperiod on mean LH concentration which approached the level of significance ($P = 0.056$). However, there was no significant effect of drug treatments on this parameter.

No effect of time was revealed either on mean LH concentration (ANOVA) or on LH pulsatility (Wilcoxon test); hormonal challenges of 12 September and 16 October were therefore pooled.

After injection of pimozide, the mean number of pulses significantly increased only in RSD ewes: 0 versus 0.9 pulses in 4 h ($P = 0.05$) and they were higher in this group than in group ILD: 0.9 versus 0.25 ($P = 0.05$). The mean number of pulses in RSD ewes was significantly different from control ewes ($P = 0.02$). The mean LH concentration was not significantly different in groups RSD and ILD.

After injection of cyproheptadine, the mean number of pulses significantly increased in RSD ewes: 0 versus 1.5 pulses in 4 h ($P < 0.03$) and differed from control ewes ($P = 0.01$). The mean number of pulses significantly increased in ILD ewes: 0 versus 0.33 pulses in 4 h ($P = 0.05$) but did not differ from control ewes. The mean number of pulses after injection in group RSD was significantly higher than in group ILD: 1.5 versus 0.33 pulses in 4 h ($P < 0.03$). In group RSD, the mean LH concentration decreased by 30% after cyproheptadine injection for one ewe. If the data of this animal were discarded, the mean LH concentration significantly increased after injection in RSD

ewes (0.7 versus 1.80 ng ml⁻¹ $P < 0.05$) but not in ILD ewes (0.53 versus 0.63 ng ml⁻¹). The mean LH concentration after injection was also significantly different in groups RSD and ILD ($P < 0.03$).

Animal behaviour after drug injections

About 3 h after pimozide injections, ataxia and akinesia symptoms occurred in about 20% of the animals. After cyproheptadine injections, respiratory depression was observed in about 40% of the animals. There was no correlation between these side effects and the LH responses to drug treatments.

Discussion

The objective of the present study was to extend previous observations that suggested that dopaminergic and serotonergic systems are implicated in the inhibition of LH release in anoestrous ewes, to a situation where the photoperiod inhibits LH activity (Meyer and Goodman, 1985; 1986; Whisnant and Goodman, 1990). We therefore investigated the role of these two systems in different photoperiodic situations when LH was inhibited, in Ile-de-France ewes.

As expected, short-day treatment of Ile-de-France ewes after three months of long-day treatment advanced the onset of LH secretion, reflecting the stimulatory effects of short days. The termination of LH secretion occurred earlier in ewes subjected again to a long-day treatment (group ILD) than in ewes maintained in a short-day treatment (group RSD), which reflects the inhibitory effect of long days. These observations have

been reported earlier (Suffolk ewes: Legan and Karsch, 1980; Ile-de-France ewes: Chemineau *et al.*, 1992).

After injections of pimozide, the number of LH pulses increased in SD and RSD ewes. The ability of pimozide to increase LH pulse frequency in ovariectomized (plus oestradiol) RSD ewes has also been reported (Kao *et al.*, 1992). Taken together these results confirmed the inhibitory role of the dopaminergic system on LH release previously shown by Meyer and Goodman (1985; 1986), Thiéry *et al.* (1989) and Havern *et al.* (1991). By contrast, pimozide had no effect on LD and ILD ewes. These results are also consistent with those of Kao *et al.* (1992). The hypothesis of these authors to explain the lack of effect of pimozide in long-day photoperiods is that the dopaminergic system could be relatively unimportant during photoperiod suppression. Consequently, they have suggested that exposure to long days activates different or additional neuronal systems from those activated by prolonged exposure to a constant short day length (Kao *et al.*, 1992). However, it also seems likely that the inhibitory effect of the dopaminergic system on LH release was more effective in long-day photoperiods than in short days.

This last hypothesis is consistent with previous data since, in ovariectomized (plus oestradiol treated) ewes, higher dopaminergic contents in long than in short days were found in the median eminence (Thiéry, 1991). Results of the present experiment are also consistent with data from golden hamsters, transferred from a stimulatory photoperiod to an inhibitory photoperiod, in which a decrease in hypothalamic dopamine and noradrenaline turnover was observed (Steger *et al.*, 1982). Thus, it is suggested that the activity of catecholaminergic neurones is regulated by photoperiod.

The ineffectiveness of pimozide (0.25 mg kg^{-1}) on LH pulsatility in anoestrous ewes maintained under natural or artificial long days was also reported (Kao and Jackson, 1990; Kao *et al.*, 1992). In anoestrous ewes in natural conditions, pimozide (0.4 mg kg^{-1}) slightly increased pulse frequency and had no effect on mean LH concentration (Curlewis *et al.*, 1991).

By contrast, Meyer and Goodman showed that pimozide increased LH pulse frequency in anoestrous ewes (Meyer and Goodman, 1985; 1986). However, the dose of pimozide used in the present study (0.08 mg kg^{-1}) was lower than that routinely used by the latter authors who showed a dose-response effect of pimozide on LH pulse frequency; hence it appeared that a dose of 0.8 mg kg^{-1} was effective in increasing LH pulse frequency. Thus, if activity of dopaminergic neurones was more efficient in long-day photoperiods a disinhibition of LH secretion with low doses appears to be impossible. However, we did not test these higher doses because of severe side-effects of the drug on our experimental animals.

Our results also suggest that serotonergic mechanisms played a major role in photoperiodic inhibition of LH pulsatile release since cyproheptadine strongly increased the number of LH pulses in SD and RSD ewes. An effect of cyproheptadine was also shown in LD ewes, but it was less marked than in the same ewes under short days (SD ewes). In ILD ewes, a small effect of cyproheptadine was reported and it was less than in RSD ewes. These results are in agreement with those obtained with pimozide, since it appeared that the disinhibition of LH release by an antagonist to serotonergic receptors was also more efficient in short- than in long-day photoperiods. On

the basis of these observations, it can be postulated that the serotonergic neural activity was also subjected to photoperiod regulation.

These results support the idea that, in Ile-de-France ewes, LH release is under the control of serotonergic systems and that long days may induce activation of the inhibitory activity of these neurones and that short days may reduce this activity. This strengthens the previous hypothesis that putative inhibitory neurones are activated by an inhibitory photoperiod and account for the effects of long days on LH secretion in ewes (Goodman and Meyer, 1984). In the same way, the dopaminergic system could be implicated in LH release in short-day photoperiod but, in long-day photoperiod, its implication is more controversial (Kao *et al.*, 1992 and present study) and will need to be confirmed in further experiments.

Another interesting point is that, because pimozide and cyproheptadine increased the number of LH pulses in ewes that had become refractory to short days (RSD ewes), short-day refractoriness could be due to an activation of both dopaminergic and serotonergic inhibitory neurones. However, because of the small number of animals in this part of the experiment, these results need to be confirmed in further experiments. In contrast, Kao *et al.* (1992) suggested a minor role of the serotonin neural mechanism since cyproheptadine (3 mg kg^{-1}) has few effects on LH pulse frequency in ovariectomized (plus oestradiol treated) RSD ewes. The reasons for this discrepancy are difficult to explain but could be due to a breed difference or a different endocrine status of the animal or to a different procedure of blood sampling. Effectively, these last authors took blood samples for 8 h after drug injections compared with 4 h in the present experiment. However, when they analysed data collected only during the first 4 h of their blood collection protocol separately, their conclusions were the same. The procedure of blood sampling for 4 h after injection of the drug therefore seems to be sufficient to detect an increase in pulse frequency. In addition, before drug injections the ewes in our study did not exhibit pulses, whereas those of these authors exhibited several pulses. In their case, it would therefore have been more difficult to detect an increase in pulse frequency. Since the ewes were not in the same physiological state, it is impossible to compare the results directly.

Our results demonstrated the inhibition by the serotonergic system of LH pulsatility in the presence of oestradiol. Until now, it was assumed that serotonergic neurones mediate the steroid-independent suppression of LH pulse frequency as it was shown that cyproheptadine (3 mg kg^{-1}) increased LH pulse frequency in ovariectomized anoestrous ewes but not in ovary intact anoestrous ewes (Meyer and Goodman, 1985); similar results were obtained using another serotonergic (5HT_2) receptor antagonist (methysergide) (Whisnant and Goodman, 1990). Ineffectiveness of cyproheptadine (3 mg kg^{-1}) to increase LH pulse frequency in anoestrous ovary-intact ewes maintained under natural or artificial long days was also reported (Kao and Jackson 1990). However, recent studies also reported the same conclusion in anoestrous ovariectomized (plus oestradiol treated) ewes (Kao *et al.*, 1992). There may be breed differences, as repeated intraperitoneal injections of a serotonin synthesis inhibitor (parachlorophenylalanine) delayed the date of cessation of ovulatory activity due to refractoriness to short days, in Ile-de-France ovary intact ewes (P. Chemineau *et al.*, 1992).

Finally, it should be pointed out that cyproheptadine, used in our experiments, has a higher affinity for histamine receptors (H_1) than for $5HT_2$ receptors (Leysen *et al.*, 1981). It has been suggested that histamine acts at the level of the central nervous system through an H_1 -receptor mechanism to control secretion of LH in ewes since an antagonist of the H_1 -receptor decreased LH in ovariectomized ewes (Van Kirk *et al.*, 1989). As a result of the antihistamine properties of cyproheptadine (Stone *et al.*, 1961), it seems unlikely that the effects of this drug on LH release in the study reported here were due to an action on H_1 -receptors. Thus, although it seems evident that the serotonergic system is involved in the control of reproductive response in ewes, its interactions with steroids or with other gonadal compounds remain to be elucidated.

In conclusion, our results support the inhibitory role of dopaminergic and serotonergic systems on LH pulsatile release and suggest that refractoriness to short days is due to the activation of both inhibitory dopaminergic and serotonergic neurones.

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