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MODULATORY ACTIONS OF THE NEW ANTIPROGESTINS ZK 98.299 AND ZK 98.734 AND OF RU 486 ON LUTEINIZING HORMONE SECRETION AND PROGESTERONE EFFECTS IN PITUITARY GONADOTROPHS*

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Summary—The effects of the antiprogestins (APs) ZK 98.299, ZK 98.734 and RU 486 on GnRH-stimulated LH secretion and their antagonistic activity on progesterone (P) actions were investigated in cultured pituitary cells from adult female Wistar rats. P (100 nM) was able to exert a facilitatory effect on GnRH (1 nM)-induced LH secretion after short-term (4 h) treatment of estradiol-primed (1 nM, 48 h) rat pituitary cells. When the APs (10 pM–10 μM) were introduced during the 4 h incubation period with P the facilitatory effect of P was totally abolished at concentrations > 10 nM (ZK 98.299, ZK 98.734) and > 1 nM (RU 486). Also the APs were shown to block the inhibitory action of P which occurs after long-term incubation of pituitary cells with this steroid. However at concentrations > 10 nM (ZK 98.734, RU 486) and > 100 nM (ZK 98.299) this antagonistic action of the APs was lost. To evaluate whether the APs have direct effects on GnRH-induced LH secretion in the absence of exogenous P pituitary cells cultivated for 48 h with or without 1 nM estradiol were incubated for 4 or 24 h with increasing concentrations of the APs (10 pM–10 μM). Four hour treatment of non-estradiol-primed cells with ZK 98.299 or ZK 98.734 was without any effect on the LH response to a 1 nM GnRH-stimulus. Only the highest concentration of RU 486 (10 μM) reduced the LH response. Twenty-four hour treatment of the cultures with the APs led to enhancement of GnRH-stimulated LH secretion by up to 113, 37 and 33% for ZK 98.734, ZK 98.299 and RU 486, respectively. When estradiol-primed cells were used for the same experiments we observed exclusively inhibitory effects on GnRH-induced LH secretion after 4 and 24 h treatment periods.

It is concluded that these new APs are potent inhibitors of P-actions, but also *per se* they induce diverse effects on GnRH-stimulated LH secretion in cultured rat pituitary cells which have to be taken into account.

INTRODUCTION

The actions of the synthetic steroid derivative RU 486 have been studied extensively *in vivo* and *in vitro*. It has been described to be a weak antiandrogen and to possess potent antiglucocorticoid and antiprogesterone activity. Due to its antiprogestational actions RU 486 is able to interrupt the luteal phase of the menstrual cycle to induce abortion and to impair gonadotrophin secretion *in vivo* [1–3]. Our group demonstrated that the latter action can be explained by a direct effect of RU 486 on gonadotrophin secretion from the pituitary gland [4]. As an antiglucocorticoid RU 486 can disrupt the negative feedback effect of glucocorticoids on ACTH secretion and has also been used in the treatment of Cushing's disease because of its peripheral antiglucocorticoid action [5–7].

In order to select more effective antiprogestins attempts have been made to dissociate antiglucocorticoid and antiprogesterone activity of the antihormone. Two new compounds with reduced antiglucocorticoid activity, ZK 98.299 and ZK 98.734, have recently been synthetized [8].

In the model of cultured rat pituitary cells we demonstrated that RU 486 blocked both the inhibitory and stimulatory actions of progesterone on GnRH-stimulated LH secretion and also showed that the antiprogestin itself induced an antigonadotrophic effect in the absence of progesterone. Referring to this we now report about comparative studies with ZK 98.299, ZK 98.734 and RU 486 that were carried out

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to evaluate whether the new compounds are superior to RU 486 and therefore might be more useful for both research and therapeutic applications. For this purpose rat pituitary cells in static culture were treated with increasing concentrations of the antiprogestins after appropriate pre- or coincubations with progesterone and estradiol to determine their ability to antagonize progesterone actions on LH secretion. To evaluate whether ZK 98.299 and ZK 98.734 can modulate GnRH-stimulated LH secretion *per se* as it was shown for RU 486, the cells were treated with different concentrations of the antiprogestins for short-term and long-term incubation periods in the absence of progesterone. Since the actions of progesterone are known to be dependent on estrogen-induced progesterone receptors pretreatments with estradiol were also performed during these experiments.

EXPERIMENTAL

Hormones

ZK 98.299 [11β-(4-dimethylaminophenyl)17-hydroxy-17β-(3-hydroxy-propyl)-13-methyl-4,9-gonadien-3-one] and ZK 98.734 [11β-(4-dimethyl-aminophenyl)-17β-hydroxy-17-(3-hydroxy-prop-1(Z)-enyl)-4,9(10)-estradien-3-one] were provided by Schering (Berlin, Germany). RU 486 [11β-(4-dimethylaminophenyl)-17β-hydroxy-17-(1-propinyl)-4,9(10)-estradien-3-one] was a gift from Roussel-Uclaf (Romainville, France). Estradiol, progesterone and GnRH were purchased from Sigma Chemical Co. (Deisenhofen, F.R.G.). The steroids were prepared in ethanol, and GnRH was dissolved in PBS containing 1 g/l BSA.

Pituitary cell preparation and culture conditions

Anterior pituitary glands obtained from adult female Wistar rats (Winkelmann, Borchen-Kirchborchen, F.R.G.) at random stages of the estrous cycle were used for the preparation of primary cultures of pituitary cells as previously described [9–11].

Before any hormone treatment the cultures were washed with freshly prepared medium. Long-term incubations (>4 h) were carried out in incubation medium (see above); the same medium containing 0.1% BSA instead of 10% horse serum was used for short-term treatment periods (<4 h). Steroids were added to the required medium from appropriate stock solutions in ethanol. Respective control cultures were exposed to medium containing the same quantity of ethanol without steroids (V = vehicle). During long-term treatment, media containing steroids were changed every 24 h. The final concentration of ethanol in the medium was 0.2%. When cells were stimulated with GnRH they were first washed and incubated with serum-free medium for 1 h, and then with fresh medium to which GnRH was added directly in 20 μl vol; control cultures received 20 μl PBS containing 0.1% BSA. After incubation of the control and GnRH-treated cell cultures for 3 h,

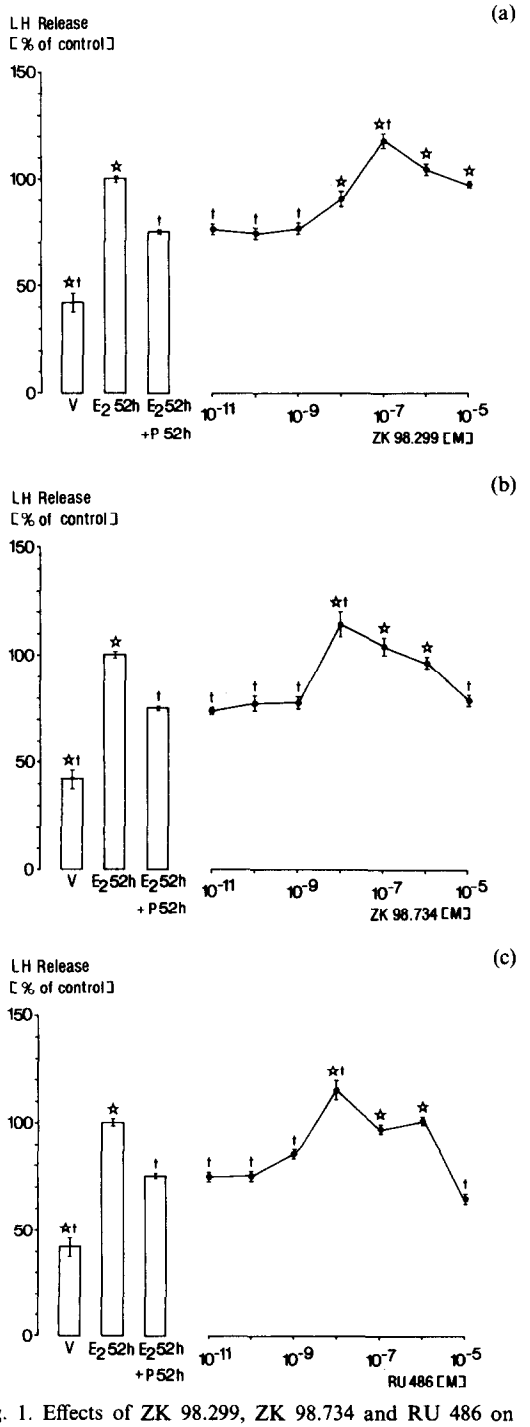

Fig. 1. Effects of ZK 98.299, ZK 98.734 and RU 486 on the long-term inhibitory action of progesterone on GnRH-induced LH release. Pituitary cell cultures were incubated for 52 h with vehicle (V, 0.2% ethanol), 1 nM estradiol (E_2) or 1 nM E_2 plus 100 nM progesterone (P) in the absence or presence of increasing concentrations of the antiprogestins ZK 98.299 (a), ZK 98.734 (b) or RU 486 (c) and stimulated with a submaximal concentration of GnRH (1 nM) during the last 3 h of the incubation period. The data from three independent experiments each performed in triplicate were pooled and expressed as percent (mean ± SE) of the LH release by the respective control cultures (E_2). The mean absolute LH value corresponding to 100% is 37 ± 6 ng/ml. †$P < 0.05$ vs E_2, *$P < 0.05$ vs E_2 + P (Newman–Keuls-test); the P value of ANOVA is <0.05.

media were removed and stored at −20°C until assayed for their LH content. Cells were checked for viability by the trypan blue exclusion method after each experiment, and none of the performed treatments was found to exert toxic effects by this criterion. All experiments were performed in triplicate, and each experiment was repeated at least twice.

Effects of the antiprogestins on progesterone-modulated LH release

To determine whether the antiprogestins ZK 98.734, ZK 98.299 and RU 486 are able to antagonize the long-term inhibitory effect of progesterone on GnRH-stimulated LH secretion, pituitary cells were incubated for 52 h with V, 1 nM estradiol or 1 nM estradiol + 100 nM progesterone in the absence or presence of increasing concentrations of the antiprogestins (10 pM–10 μM). The actions of the antiprogestins on the short-term facilitatory effect of progesterone were investigated in pituitary cells that had been treated for 52 h with 1 nM estradiol and received 100 nM progesterone with or without increasing concentrtions of ZK 98.299, ZK 98.734 or RU 486 during the last 4 h of the incubation period. In each case the cells were stimulated with a submaximal concentration of GnRH (1 nM) during the last 3 h of the indicated treatment periods.

Effects of the antiprogestins on LH release in the absence of progesterone

To check whether the antiprogestins can modulate GnRH-stimulated LH secretion in the absence of progesterone, pituitary cells were incubated for 4 or 24 h with increasing concentrations of ZK 98.299, ZK 98.734 or RU 486 (10 pM–10 μM) and stimulated with 1 nM GnRH during the last 3 h of the incubation periods. As RU 486 has been shown to inhibit LH secretion an action that is dependent on estradiol-priming of the pituitary cells these experiments were carried out using cells that had been pretreated for 48 h with V or 1 nM estradiol.

RIA and data analysis

LH content of the incubation media was determined by RIA using the reference preparation RP-2 (AFP-5666 C) provided by the National Pituitary Agency, Baltimore, Md. [12]. The LH release by different treatment groups was expressed in terms of percentage of LH release by the respective control groups (control = 100%). The data obtained in three different experiments run in triplicate each were pooled. Statistical analysis was performed as follows: first the data were tested for homogeneity of variance using the Bartlett-test. Then analysis of variance (ANOVA) was carried out. Statistical significant differences between individual groups were determined by the Newman–Keuls-test. If variances were not homogenous data were analyzed for statistical

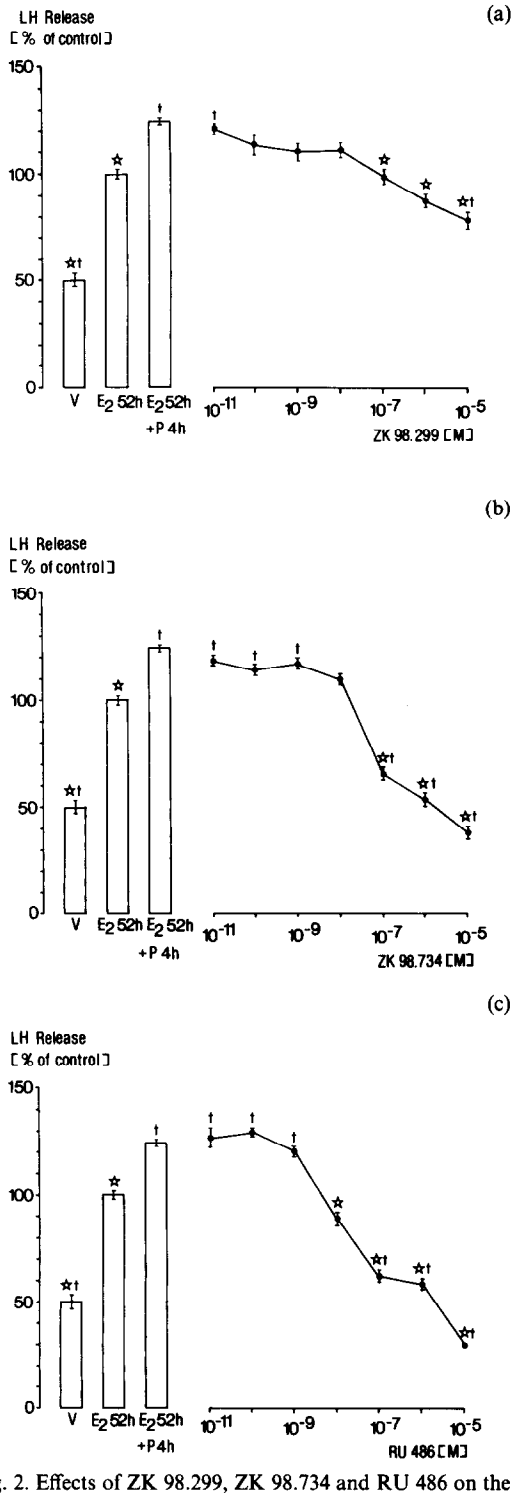

Fig. 2. Effects of ZK 98.299, ZK 98.734 and RU 486 on the short-term facilitatory action of progesterone on GnRH-induced LH release. Pituitary cell cultures were incubated with V for 52 h or pretreated with 1 nM E_2 for 52 h and treated with 100 nM P with or without increasing concentrations of the antiprogestins ZK 98.299 (a), ZK 98.734 (b) or RU 486 (c) during the last 4 h of the incubation periods. GnRH-stimulation (1 nM) was performed during the last 3 h of the treatment periods. The mean absolute LH value corresponding to 100% is 32 ± 3 ng/ml. †$P < 0.05$ vs E_2, *$P < 0.05$ vs E_2 + P (Newman–Keuls-test); the P value of ANOVA is <0.05. For further details see Fig. 1.

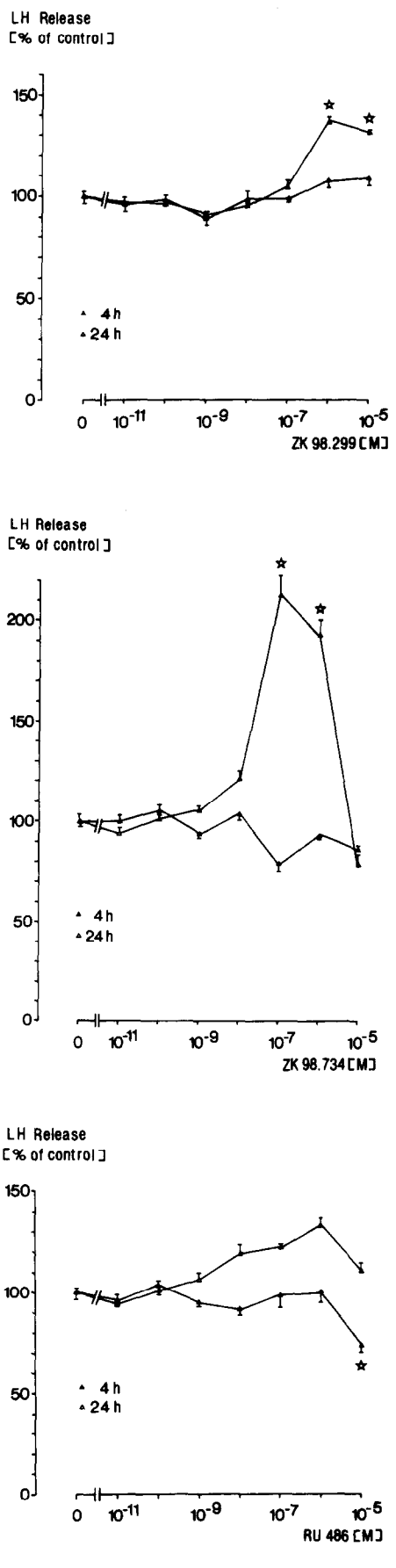

Fig. 3. Short-term and long-term effects of ZK 98.299, ZK 98.734 and RU 486 on GnRH-induced LH release. Pituitary cell cultures were incubated for 4 or 24 h with increasing concentrations of the antiprogestins ZK 98.299 (a), ZK 98.734 (b) or RU 486 (c) and stimulated with 1 nM GnRH during the last 3 h of the incubation periods. Mean absolute LH values corresponding to 100% are 13 ± 1 ng/ml (4 h) and 14 ± 1 ng/ml (24 h). $^*P < 0.05$ vs control (Newman–Keuls- or Nemenyi-test); the P values of ANOVA or Kruskall–Wallis-test are <0.05. For further details see Fig. 1.

significant differences using the Kruskall–Wallis-test followed by a Nemenyi-test for comparison of individual groups.

RESULTS

Effects of the antiprogestins on progesterone-modulated LH release

When pituitary cells were incubated for 52 h with 1 nM estradiol plus 100 nM progesterone the sensitizing effect of estradiol on GnRH-induced LH secretion was partially reduced by 25%. Increasing concentrations of ZK 98.734, ZK 98.299 or RU 486 were able to reverse this inhibitory action of progesterone. Complete antagonism was achieved at concentrations of >1 nM, 10 nM and >10 nM for RU 486, ZK 98.734 and ZK 98.299 respectively. At concentrations higher than 10 nM (RU 486, ZK 98.734) and 100 nM (ZK 98.299) the antagonistic action of the antiprogestins got lost and the LH release of pituitary cells that had been treated for 52 h with 1 nM estradiol plus 100 nM progesterone plus 10 μM RU 486 or ZK 98.734 was not different from LH release by cells that had been incubated with estradiol plus progesterone alone (Fig. 1).

Short-term progesterone treatment (100 nM, 4 h) of pituitary cells that had been primed with 1 nM estradiol (48 h) induced a facilitatory action on GnRH-stimulated LH release when compared to estradiol treatment alone. Coincubation of the cells with the antiprogestins during the 4 h incubation period with progesterone blocked its facilitatory action. It was fully antagonized at concentrations >1 nM, >10 nM and >10 nM for RU 486, ZK 98.734 and ZK 98.299, respectively. Higher concentrations of the antiprogestins even suppressed LH release below the respective control level (Fig. 2).

Effects of the antiprogestins on LH release in the absence of progesterone

When we used pituitary cells that had not been primed with estradiol for dose–response studies of the antiprogestins we found that short-term incubation with ZK 98.299 or ZK 98.734 (10 pM–10 μM) had no effect while RU 486 inhibited GnRH-induced LH secretion by 27% at a concentration of 10 μM. Long-term incubation of non-primed cells with RU 486 or ZK 98.299 led to moderate augmentation

of GnRH-stimulated LH release at concentrations >10 nM whereas ZK 98.734 showed a dramatic sensitisation of the cells to the 1 nM GnRH stimulus. This effect was present at concentrations of 100 nM and 1 μM with a maximal enhancement of GnRH induced LH release by up to 213% and was lost at 10 μM (Fig. 3).

When the same experiments were carried out with cells that had been primed for 48 h with 1 nM estradiol we observed exclusively inhibitory effects for all antiprogestins. Also there were no major differences between short-term and long-term incubation (Fig. 4).

DISCUSSION

In the present study it was clearly demonstrated that all of the tested antiprogestins were able to block the well established inhibitory and stimulatory effects of progesterone on GnRH-induced LH secretion in cultured rat pituitary cells. The order of potency concerning the antagonistic effect of the antiprogestins was RU 486 > ZK 98.734 > ZK 98.299. When we used the antiprogestins to reverse the long-term inhibitory action of progesterone on LH secretion we observed that the lower concentrations dose-dependently blocked this action whereas the higher concentrations paradoxically were not able to antagonize the progesterone effect. Also all antiprogestins blocked the facilitatory action of progesterone and suppressed GnRH-stimulated LH secretion not only to the level of the controls but even below it. Although such an overshoot is sometimes observed when antihormones are used, one might also claim that this effect is due to an action of the antiprogestins that can be dissociated from their antagonistic activity [13]. Therefore experiments with the antiprogestins were carried out in the absence of progesterone to check whether these compounds modulate GnRH-induced LH secretion *per se*. In cells that had not been primed with estradiol the antiprogestins induced a stimulatory effect on GnRH-stimulated LH release after 24 h treatment. In this respect ZK 98.734 was shown to be very potent. Short-term treatment was without an effect except for 10 μM RU 486 which inhibited LH secretion. Although the concentrations of the antiprogestins needed to induce these effects are quite high one has to consider that the therapeutic doses for example for induction of abortion are also very high, and concentrations in the micromolar range are attained *in vivo* [Ref. cf. [4]). In estradiol-primed cells all the antiprogestins showed exclusively inhibitory actions. These results may indicate that this effects of these compounds are dependent on the amount of estrogen-induced progesterone receptors. In our previous study we were not able to demonstrate a stimulatory effect of RU 486 on LH secretion after long-term treatment of non-estrogen-primed cells which was most likely due to the fact that we did

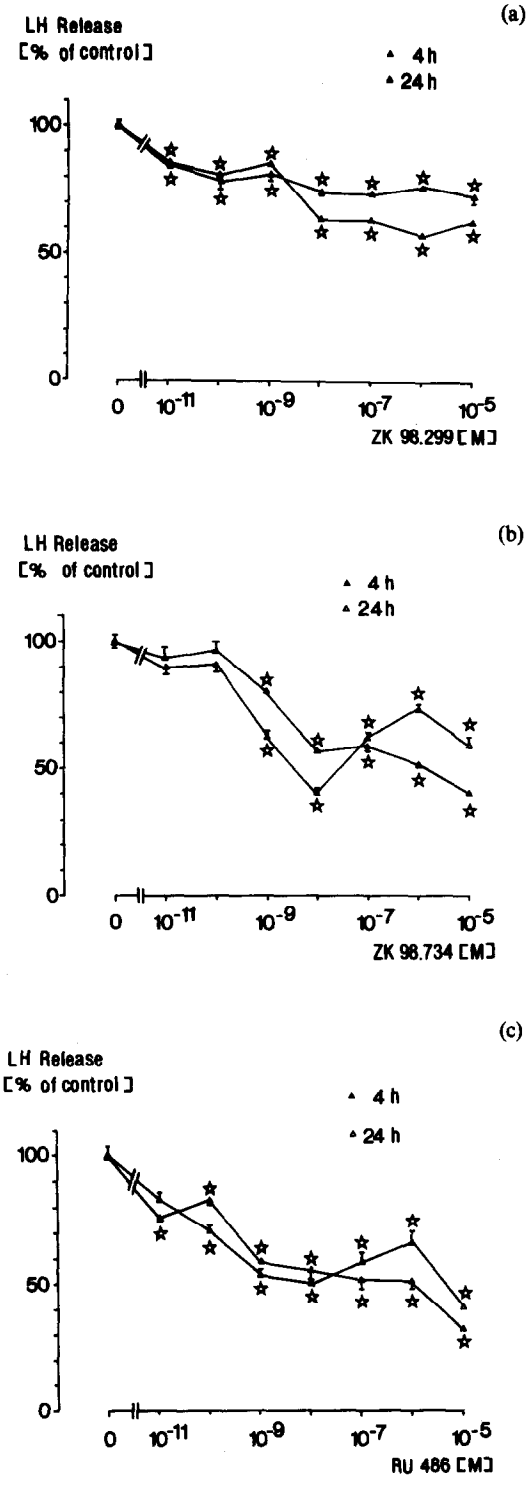

Fig. 4. Short-term and long-term effects of ZK 98.299, ZK 98.734 and RU 486 on GnRH-induced LH release from estradiol-primed rat pituitary cells. Pituitary cell cultures that had been primed for 48 h with 1 nM E_2 were incubated for 4 or 24 h with increasing concentrations of the antiprogestins ZK 98.299 (a), ZK 98.734 (b) or RU 486 (c) and stimulated with 1 nM aGnRH during the last 3 h of the incubation periods. Mean absolute LH values corresponding to 100% are 27 $\pm$ 1 ng/ml (4 h) and 33 $\pm$ 2 ng/ml (24 h). *$P < 0.05$ vs control (Newman–Keuls-test); the P value of ANOVA is <0.05. For further details see Fig. 1.

not establish estrogen-free conditions as the culture media contained phenol red which is known to possess estrogenic activity [11, 14].

The data derived from experiments in which we tested the effects of the antiprogestins in the absence of progesterone revealed a number of time-, dose- and estrogen-dependent modulatory actions of the three compounds on GnRH-induced LH secretion. Although the antiprogestins might possess partial agonist activity as described in the endometrium such activity cannot explain the direct actions observed in the present study because these do not mimic the modulatory effects of progesterone on gonadotrophin secretion [15]. Interpretation of the results is further complicated as the tested compounds do not only bind to the progesterone receptor but also to the glucocorticoid and androgen receptor [16, 17]. However the negative actions on gonadotrophin secretion which occur after estradiol pretreatment indicate that this effect is more likely mediated via the progesterone receptor.

The direct actions of the antiprogestins have to be taken into account when interpreting the antagonistic effects of the compounds. Although all of the antiprogestins effectively blocked the facilitatory action of progesterone, the effective concentrations also induced an inhibition of GnRH-stimulated LH secretion *per se*. Therefore the present data do not allow a clear conclusion concerning the antagonistic action of the antiprogestins on the facilitatory progesterone effect. Also our findings put those studies into question in which RU 486 was applied to women or subhuman primates during the follicular phase of the menstrual cycle, and conclude that progesterone is essential for the induction of the preovulatory LH surge because such treatment caused a delay of the surge [18–21]. However, the present and previous data revealed the antigonadotrophic activity of the antiprogestins, which might also explain the observed effect and puts the above interpretation into question [2, 4]. A recent study, being aware of this problem, has shown that doses of RU 486 that are not high enough to suppress gonadotrophin secretion in women are well able to delay the midcycle LH surge [22]. As to the antagonism of the negative progesterone effect the situation is quite different because long-term incubation of pituitary cells with the antiprogestins alone leads to inhibition of GnRH-stimulated LH secretion while LH release is enhanced even above the respective control values (estradiol + progesterone for 48 h) when the cell cultures were incubated with estradiol + progesterone + antiprogestin. However at the higher concentrations >10 nM (RU 486, ZK 98.734) and >100 nM (ZK 98.299) the antagonistic action of the antiprogestins is reversed. This phenomenon can possibly be explained by the pronounced inhibitory effect of the antiprogestins on LH secretion at these concentrations. One could also speculate that the dual action of the antiprogestins (antagonism of progesterone effects/inhibition of LH secretion) might be mediated via the same receptor exerting different cellular responses depending on the concentration ratio between progesterone and antiprogestin or two distinct binding sites of which one might be the progesterone receptor and the other an antiprogestin binder which has recently been described for RU 486 [23].

Although the present data clearly characterize the actions of three antiprogestins under different conditions the underlying molecular mechanisms are not fully understood. A number of recent studies showed, that RU 486 like other antisteroid hormones, for example antiestrogens of the triphenylethylene type, leads to impaired transformation and/or translocation processes of the steroid receptor [24–25]. There is also evidence that these processes are impaired by an interaction between RU 486 and the hormone binding subunit of the progesterone receptor that leads to inhibition of the dissociation of the 90 kDa heat shock protein (hsp 90) from the nontransformed steroid receptor [26]. Presumably the antisteroid is not capable to stabilize the total amount of receptors in the heterooligomeric 8S form associated to hsp 90 which could possibly explain the mixed agonist/antagonist activity of the antihormone.

In conclusion all of the antiprogestins induce inhibitory and stimulatory effects on GnRH-stimulated LH secretion *per se*. These actions are dependent on the duration of treatment, estrogen-priming and the type of antiprogestin. Concerning the antagonistic activity of the compounds RU 486 seems to be more potent than ZK 98.734 and ZK 98.299 which is less active. However ZK 98.299 may be more useful for certain purposes because it has the weakest antigonadotrophic effect after short-term treatment. Furthermore our findings demonstrate that caution should be taken when interpreting results from studies with the antiprogestins employed here.

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