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EFFECT OF A NON-PEPTIDE NK-2 TACHYKININ RECEPTOR ANTAGONIST ON LH, FSH, AND PROLACTIN RELEASE BY RAT HEMIPITUITARIES *IN VITRO* *

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Tachykinins are present in the anterior pituitary gland and there is evidence that they may have a direct intrapituitary role influencing the secretion of some of the hormones released by this gland. In this investigation, we have studied the effect of the non-peptide NK-2 receptor antagonist SR 48,968 (Sanofi Recherche) on the basal release of LH, FSH, and prolactin by rat hemipituitaries incubated *in vitro*, and also on the response to GnRH. SR 48,968 significantly inhibited prolactin release into the medium. The highest doses of this compound stimulated the basal release of LH by hemipituitaries from castrated, castrated testosterone-treated, and ovariectomized estradiol-treated rats, but not from intact male rats. SR 48,968 significantly inhibited the release of LH in response to GnRH. Since some tachykinin receptor antagonists have been demonstrated to act also on calcium channels, studies with verapamil, a calcium channel antagonist, were also carried out for comparison. Verapamil inhibited prolactin release into the medium and decreased the LH response to GnRH. These results suggest that tachykinins that bind NK-2 receptors, may have an intrapituitary role stimulating the release of prolactin, and that they may also modulate the response of the gonadotrophs to GnRH. The fact that verapamil shares some of the actions exerted by NK-2 receptor antagonists on the pituitary glandm however, suggests the possibility that some of the effects of NK-2 receptor antagonists may be mediated through calcium channel antagonism. Therefore, the results observed with the use of some of these antagonists should be interpreted with great caution.

Key words: *anterior pituitary, neurokinin A, substance P, SR 48,968, castration, tachykinins.*

INTRODUCTION

Substance P (SP) and neurokinin A (NKA) are present in the anterior pituitary (AP) gland, although in quantities much smaller than in the

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hypothalamus and in the central nervous system in general (1—4). These two tachykinins can be synthesized in the AP, since this gland contains the mRNAs that encode the synthesis of preprotachykinins, which are precursors of both SP and NKA (1, 5, 6). Tachykinins can affect the release of AP hormones when administered intracerebroventricularly (7—9). It has also been reported that tachykinins can directly affect the secretion of AP hormones, although conflicting findings exist as well (7, 10). Since the AP gland contains tachykinins, one possible approach to study the intrapituitary role of these peptides on the release of adenohypophysial hormones is through the use of tachykinin receptor antagonists *in vitro*. If tachykinins are released by AP cells, most probably by somatotrophs and thyrotrophs (11), they may exert a paracrine influence on the secretion of other hormones within the gland. Receptors binding SP, called NK-1, have been demonstrated in gonadotrophs and mammotrophs in the rat AP (12). If this paracrine effect indeed exists, it should be blocked by incubating AP glands *in vitro* in the presence of tachykinin antagonists, possibly resulting in an altered release of gonadotropins and prolactin. In recent years several potent non-peptide tachykinin antagonists have been synthesized (13, 14). One of the most powerful compounds acting on NK-2 receptors, which are activated by NKA, is a substance named with the code SR 48,968 (hereafter referred to as SR) (15). It was then considered of interest to study the effects of this NK-2 receptor antagonist on the release of gonadotropins and PRL by hemipituitaries *in vitro*. Since steroid hormones may influence the response to tachykinins (8—10), and therefore most likely also to their antagonists, AP glands from intact, castrated, and castrated, steroid-treated rats were used for these studies. However, in addition to their effects on tachykinin receptors, several of these non-peptide antagonists have been shown to be potent Ca^{2+} channel antagonists as well (16, 17). In particular, the NK-2 antagonist used in the present investigation has also recently been shown to be an active Ca^{2+} channel antagonist (18). Since this compound may be a useful research tool, and also it may have some therapeutic potential, it is essential to define whether the effects of this antagonist could be due, to some extent, to its Ca^{2+} channel antagonistic activity. To this purpose, comparative *in vitro* incubations with the well known Ca^{2+} channel antagonist verapamil (16) were carried out.

MATERIALS AND METHODS

Drugs

SR 48,968 (SR) was obtained from Sanofi Recherche, Montpellier, France, in pure powder form, through the courtesy of Dr. X. Emonds-Alt. Since this compound is more readily soluble in organic solvents than water, a small amount was weighed and immediately before the incubations dissolved in 96% ethanol and then diluted 1 : 10 with the incubation medium to a concentration of

1 μg/μl. Aliquots of this solution were added to the incubation medium at 3 dose levels: 100 ng/ml, 1 μg/ml, and 10 μg/ml.

SR 48,965, an (R)-inactive enantiomer of SR 48,968 (EN), which was tested in some control studies as well, was also obtained from Sanofi Recherche, and diluted as described for SR, to be used at a 10 μg/ml concentration.

Verapamil was obtained from Sigma Chemical Co. (St. Louis, MO). It was dissolved in saline and used in incubations of anterior pituitary halves at 100 ng, 1 μg, and/or 10 μg/ml dose levels.

Animals

Young adult rats (mean weight: 250 g for males and 200 g for females) of the Sprague-Dawley strain (Harlan-Sprague-Dawley, Indianapolis, IN)) were used throughout the investigation.

Incubation media

For all the incubations, medium TC-199 plus HEPES (Sigma Chemical Co.) was gassed with carbogen for 30 min. Then, bovine serum albumin (BSA, Sigma Chemical Co.) (0.1%) and bacitracin (Fluka Chemie AG, Buchs, Switzerland) (20 μM) were added and dissolved into the medium.

Incubation procedures

The rats were killed by decapitation, the APs were exposed and cut in halves. One hemipituitary was placed in each tube containing 0.5 ml of medium. Contralateral hemipituitaries were always included in a different treatment group. Five to 6 hemipituitaries were included in each experimental group. The incubations were carried out in a Dubnoff metabolic Shaker at 37°C, at 50 strokes per min, under constant gassing with a mixture of 5% CO_2 and 95% O_2. After a preincubation of about 1 hr, the medium was discarded and replaced by fresh medium (0.5 ml/tube) containing different doses of the substances to be tested. The incubations proceeded for 3 hr, and at the end of the incubations from each tube was aspirated and kept frozen until assayed.

Experimental groups

a) SR 48,968

In the first experiment, hemipituitaries from intact, castrated, and castrated, testosterone-treated male rats were used. The time period since castration was 14 days. In the groups treated with testosterone, this androgen was given in a 1.5 cm-long Silastic capsule, filled with testosterone powder (Sigma Chemical Co., St. Louis, MO), and implanted sc immediately after castration. The hemipituitaries from the 3 groups of rats were incubated in the presence of the following substances: vehicle (control group), SR 100 ng/ml, SR 1 μg/ml, or SR 10 μg/ml.

In an additional, complementary experiment, the possible effect of SR on the AP response to gonadotropin releasing hormone (GnRH, 20 ng/ml) was studied using hemipituitaries from intact male rats. Four treatment groups were included in this incubation: control (vehicle was added), SR (10 μg/ml), GnRH (20 ng/ml), and GnRH plus SR at the same dose levels used for the previous groups.

In the third experiment, hemipituitaries from ovariectomized, and ovariectomized, estradiol-treated rats were used. The interval between ovariectomy and decapitation was 14 days, and in the estradiol-treated rats, this steroid had been given in 1.5 cm-long Silastic capsules, filled

with estradiol powder (Sigma Chemical Co.) and implanted sc immediately after ovariectomy. For the incubations, a control group (vehicle only was added to the medium), and 3 groups with different doses of SR (100 ng, 1 μg, and 10 μg/ml) were used. In this experiment, intact female rats were not used in order to avoid possible differences in sensitivity due to different stages of the estrous cycle. The incubations were carried out as indicated in the previous experiments.

b) Verapamil and EN

Verapamil was incubated with hemipituitaries from intact male rats, under conditions like those previously described, at three dose levels (100 ng, 1 μg, and 10 μg/ml). Prolactin and LH were measured in the media. In a second incubation, also using hemipituitaries from intact male rats, the effects of verapamil (10 μg/ml) on the response to GnRH were investigated. The first group of hemipituitaries was incubated with the vehicle and was considered as control, the second group was incubated with verapamil, the third group was incubated with GnRH (20 ng/ml), and the fourth group was incubated with both GnRH and verapamil, at the same dose levels already mentioned. The conditions of the incubations were the same as previously described. In the media from this incubation only LH was measured. In a third incubation, hemipituitaries from intact and castrated male rats were used. The effect of the simultaneous incubation with both verapamil and SR was studied, to investigate whether verapamil could modify the response to SR. In this incubation, the possible effect of EN was also tested at a dose of 10 μg/ml. This (R)- enantiomer of SR was previously demonstrated to be almost inactive, indicating that the binding of SR is stereoselective (15). Since it was important to define the effects of these two compounds and although these studies were designed to investigate only modifications in gonadotropin and PRL release, in this particular study GH was also determined. It was considered of importance to extend these control studies to an additional pituitary hormone, which could also be affected by SR, verapamil, and/or EN.

An additional incubation using hemipituitaries from intact male rats was run to test whether EN could modify the release of LH in response to GnRH. The conditions of the incubation and the dose of EN and GnRH were the same as previously described.

Assays

The levels of LH, FSH, PRL and/or GH in the media were determined by double-antibody radioimmunoassays using kits distributed by Dr. A. F. Parlow and the National Hormone and Pituitary Program, N.I.H. The tracers were prepared by labeling the hormones with ^{125}I using the chloramine T or Iodogen methods. rLH-RP3, rFSH-PR2, rPRL-RP2 and rGH-RP3 were used as standard preparations.

Statistics

The significance of the differences between groups was calculated by means of analysis of variance and Dunnett's test using a computer program for the Macintosh.

RESULTS

a) SR 48,968

In the first experiment (*Fig. 1*), none of the doses of SR significantly affected the release of LH or FSH by hemipituitaries of intact male rats, although the highest dose (10 μg/ml) showed some (nonsignificant) tendency to stimulate the release of LH. SR, at 1 and 10 μg/ml dose levels induced a significant decrease

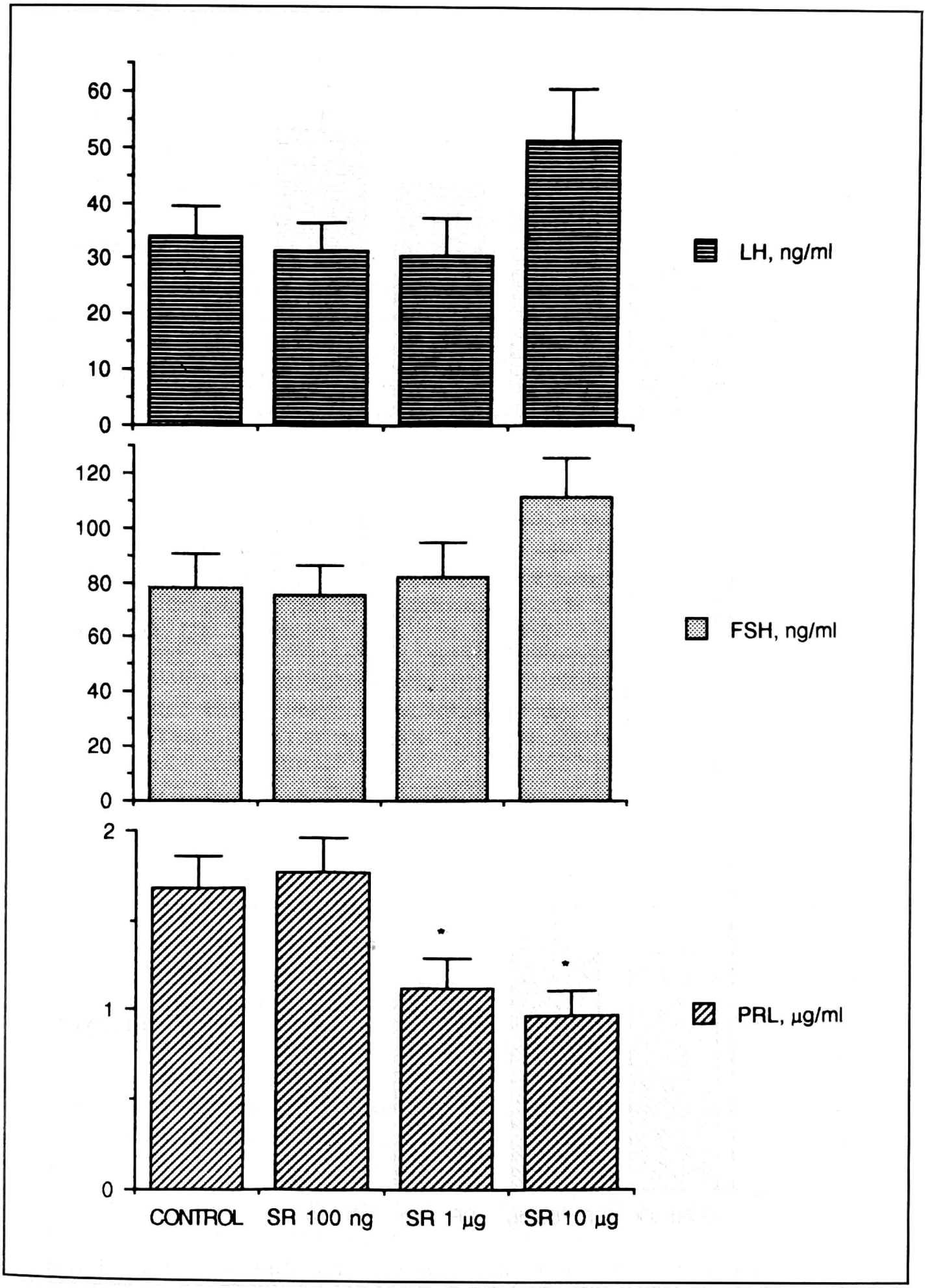

Fig. 1. Effect of SR (100 ng, 1 and 10 µg/ml) on LH (upper panel), FSH (medium panel), and PRL (lower panel) release by hemipituitaries from intact male rats. Number of samples per group: 6. * $p < 0.05$ vs. Control.

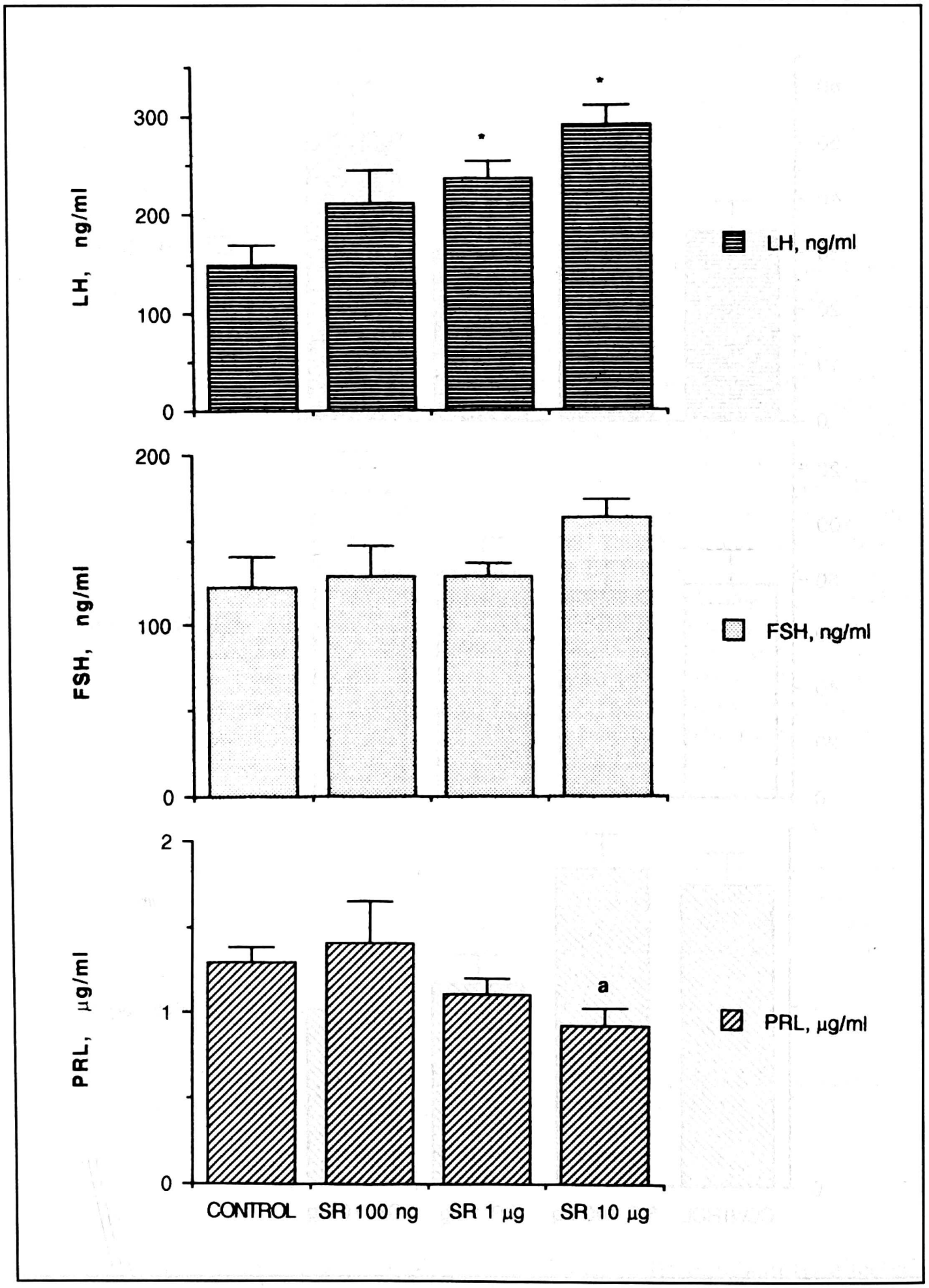

Fig. 2. Effect of SR (same doses as in *Fig. 1*) on the release of LH, FSH, and PRL by hemipituitaries from castrated male rats. Number of samples per group: 6. * $p < 0.05$ vs Control. [a] $p < 0.05$ vs SR 100 ng/ml.

of PRL levels ($p < 0.05$), as compared with the levels in the control group. In the incubation of hemipituitaries from castrated male rats (*Fig. 2*), SR, at the 1 and 10 μg/ml dose levels, induced a significant release of LH ($p < 0.05$) but not of FSH. The 10 μg/ml dose of SR induced a significant decrease of the release of PRL into the medium ($p < 0.05$) as compared with the effect of the lower SR doses. In the incubations of hemipituitaries from castrated male rats treated with testosterone (*Fig. 3*), the highest dose of SR (10 μg/ml) induced a significant decrease of PRL and a significant increase of LH ($p < 0.05$) as compared with the levels in the control group.

In the second experiment with hemipituitaries from intact male rats (*Fig. 4*), SR (10 μg/ml) induced an apparent but non-significant increase of LH release, did not affect FSH release, and decreased PRL release ($p < 0.05$), but did not affect PRL release. The release of LH and FSH from the hemipituitaries incubated with GnRH and SR was significantly higher than in the control group ($p < 0.05$), but significantly lower than in the group incubated with LH-RH alone ($p < 0.05$). In both groups incubated with SR, with or without GnRH, PRL release was significantly decreased ($p < 0.05$).

In the third experiment (*Fig. 5*), in the incubations of hemipituitaries from ovariectomized rats, SR did not affect the release of LH or FSH at any of the dose levels tested, but the 1 and 10 μg/ml doses significantly reduced PRL release ($p < 0.05$). In the incubations of hemipituitaries from ovariectomized, estradiol-treated rats (*Fig. 6*), however, all 3 doses of SR induced significant increases of LH ($p < 0.05$), but not of FSH or PRL release.

b) Verapamil and EN

Verapamil did not significantly modify the basal release of LH (*Fig. 7*), but did decrease prolactin release into the medium ($p < 0.05$). Verapamil partially, but significantly, decreased the LH response to GnRH ($p < 0.05$) (*Fig. 8*).

In the third incubation, using hemipituitaries from intact male rats, verapamil slightly decreased and SR slightly increased LH release to the medium, but the modifications were not statistically significant (*Fig. 9*). The simultaneous incubation with both verapamil and SR did not produce any significant difference in LH release as compared with the control group (*Fig. 9*). Likewise, EN was inactive in inducing any significant difference in LH release as compared with the previous groups. The release of PRL was significantly inhibited by SR and verapamil ($p < 0.05$), and when both substances were incubated together, the effect was more marked, although without reaching statistical significance compared with the effects produced by SR or verapamil separately. EN also induced significant decrease of PRL release ($p < 0.05$) (*Fig. 9*). The release of GH was stimulated by verapamil, SR, or both ($p < 0.05$) but among these three group the differences were not statistically significant. EN did not significantly modify the release of GH.

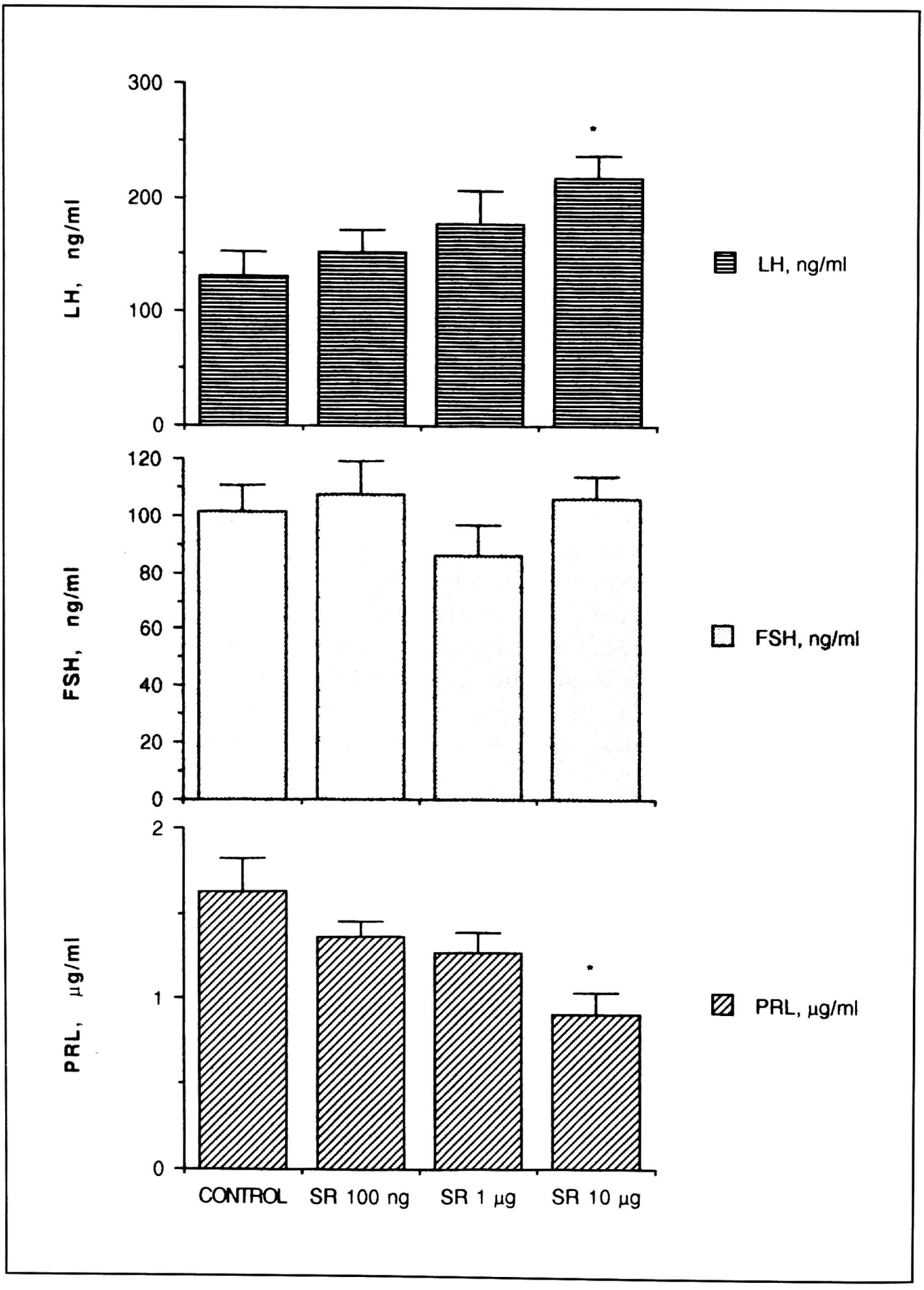

Fig. 3. Effect of SR (same doses as before) on the release of LH, FSH, and PRL by hemipituitaries from castrated, testosterone-treated male rats. Number of samples per group: 5—6. * $p < 0.05$ vs Control.

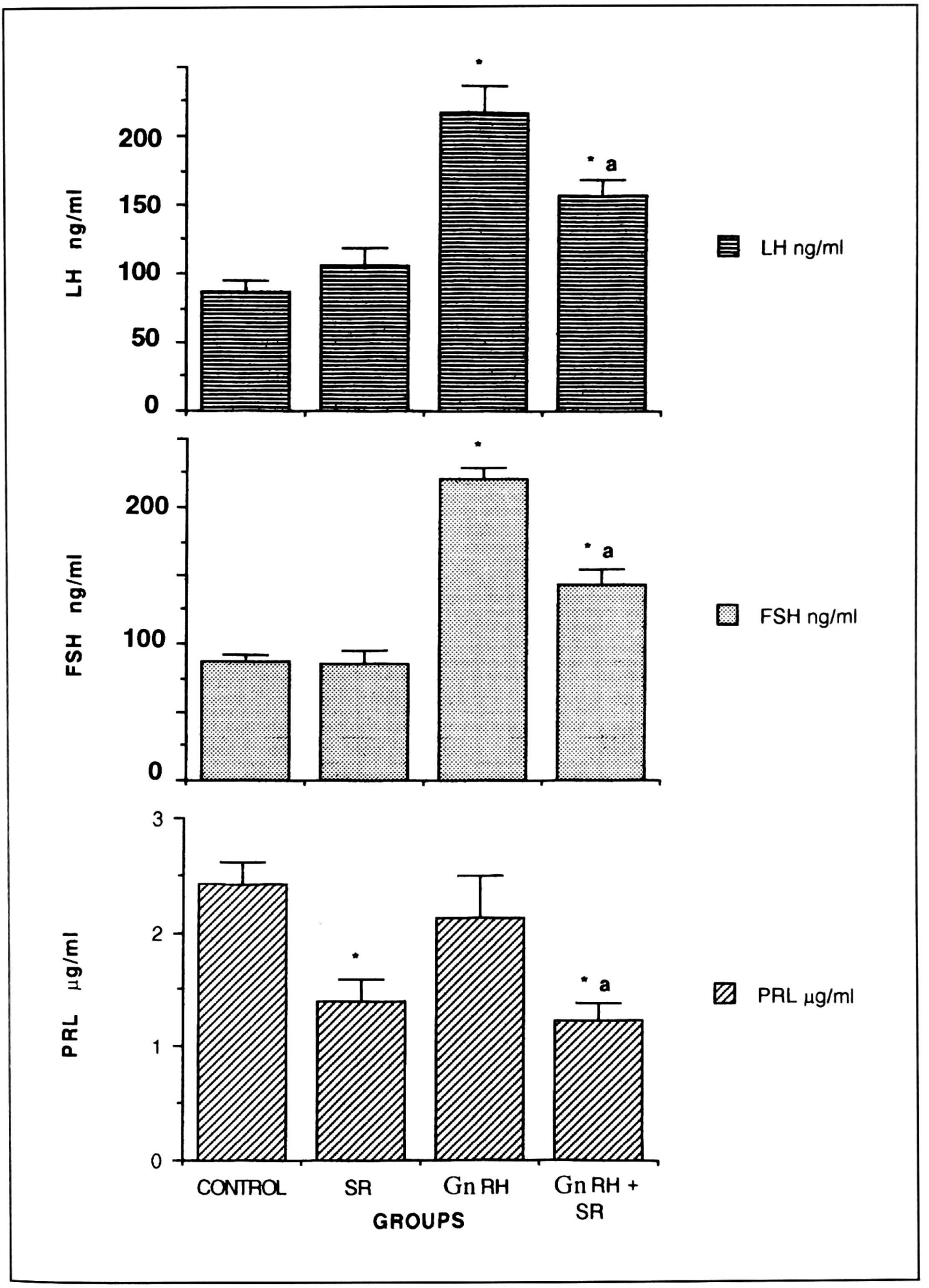

Fig. 4. Effect of SR (10 µg/ml) on the response to GnRH by hemipituitaries from intact male rats. Number of samples per group: 6. * $p < 0.05$ vs Control. [a] $p < 0.05$ vs GnRH.

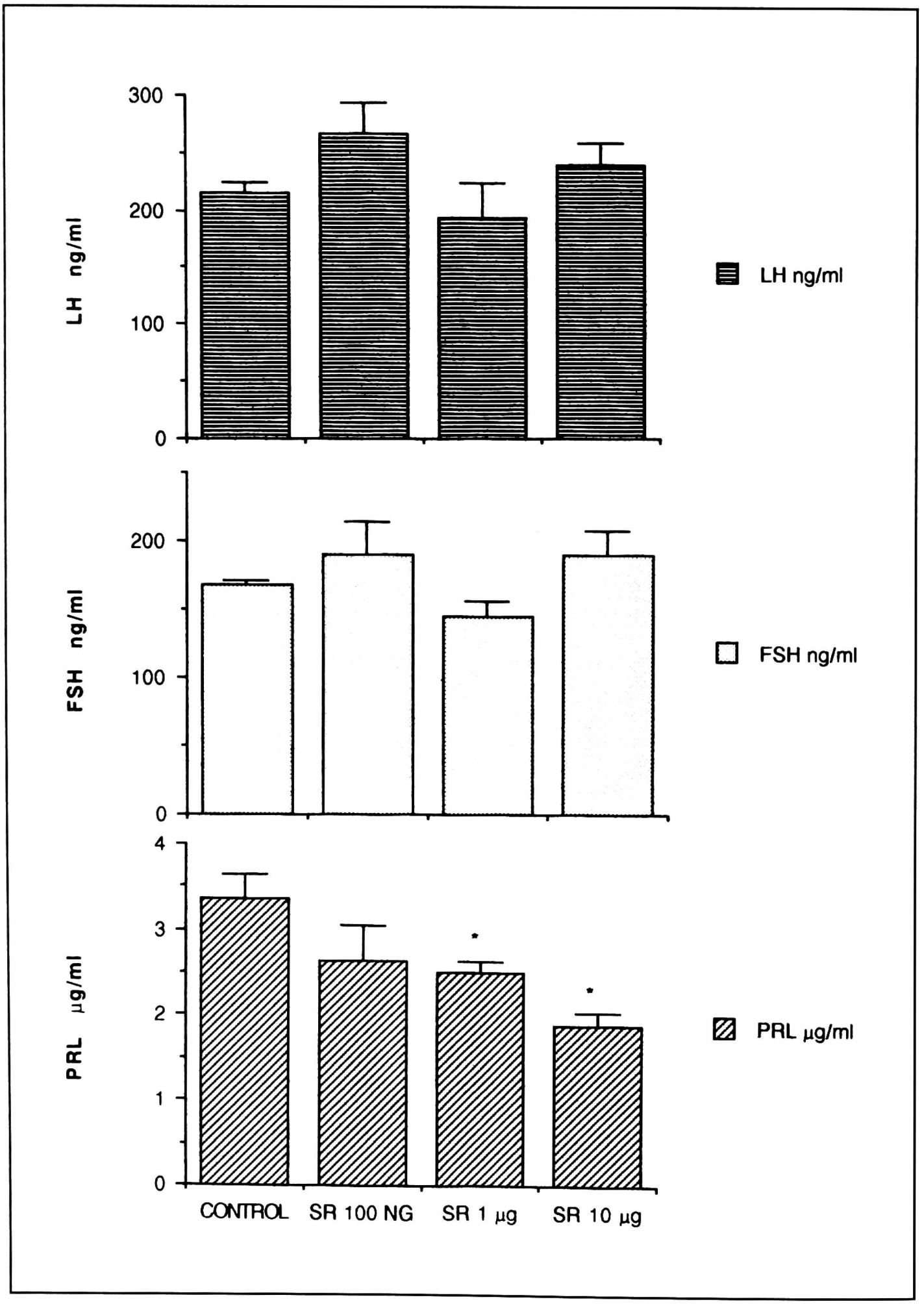

Fig. 5. Effect of SR (same doses as in *Fig. 1*) on the release of LH, FSH, and PRL by hemipituitaries from ovariectomized rats. Number of samples per group: 5. * $p < 0.05$ vs Control.

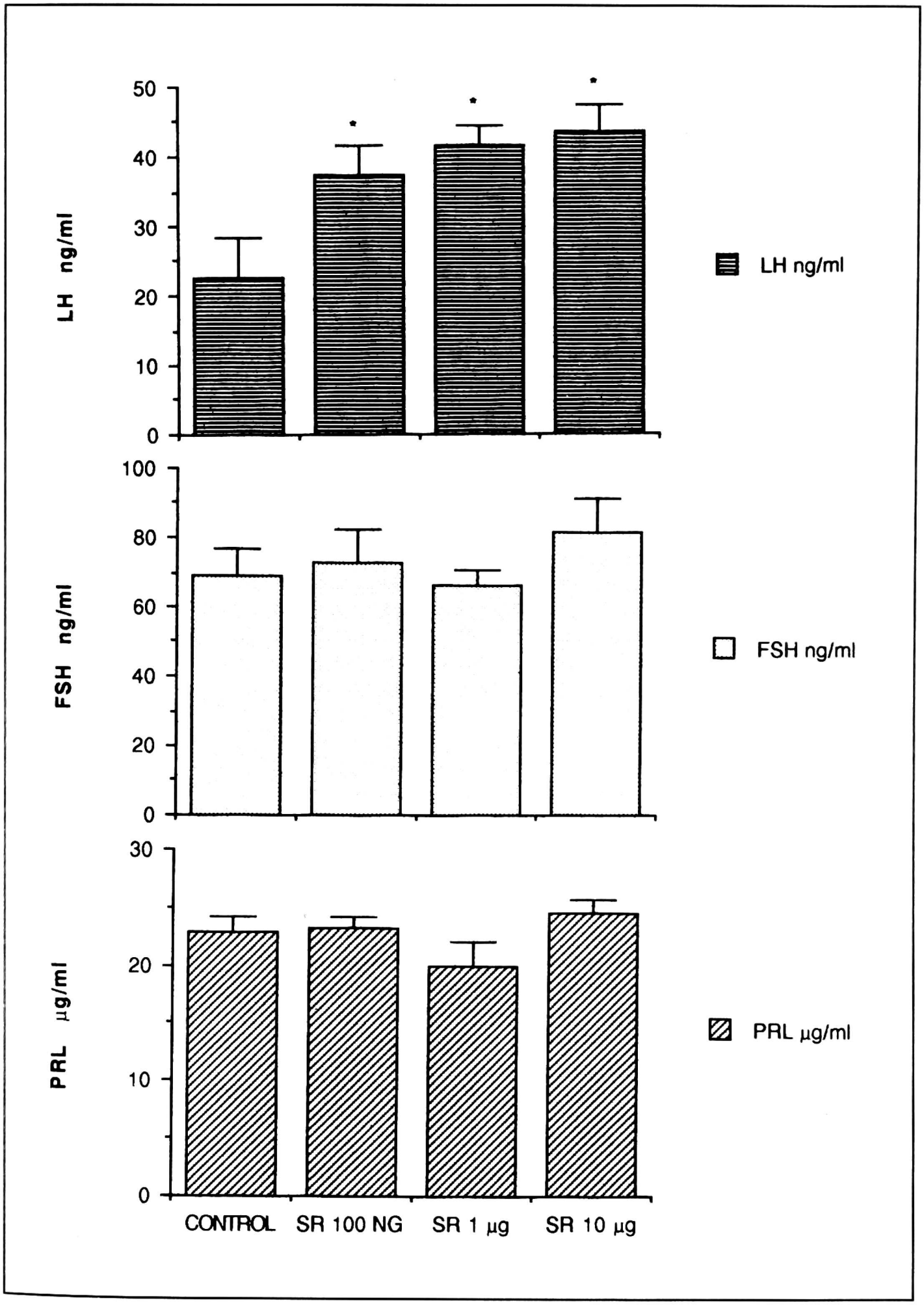

Fig. 6. Effect of SR (same doses as in *Fig. 1*) on LH, FSH and PRL release by hemipituitaries from ovariectomized estradiol-treated rats. Number of samples per group: 4—5. * $p < 0.05$ vs Control.

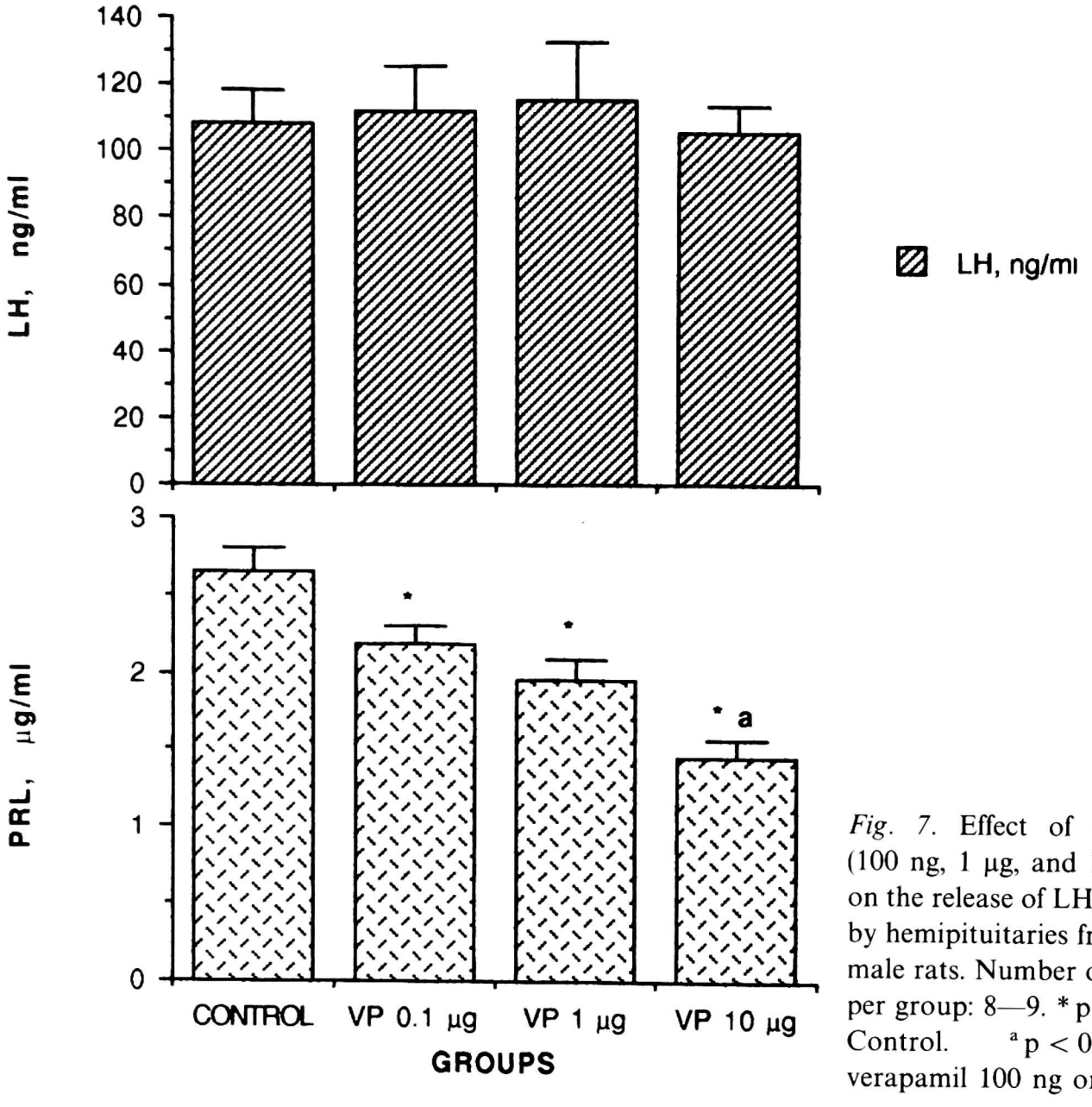

Fig. 7. Effect of verapamil (100 ng, 1 µg, and 10 µg/ml), on the release of LH and PRL by hemipituitaries from intact male rats. Number of samples per group: 8—9. * $p < 0.05$ vs Control. [a] $p < 0.05$ vs verapamil 100 ng or 1 µg/ml.

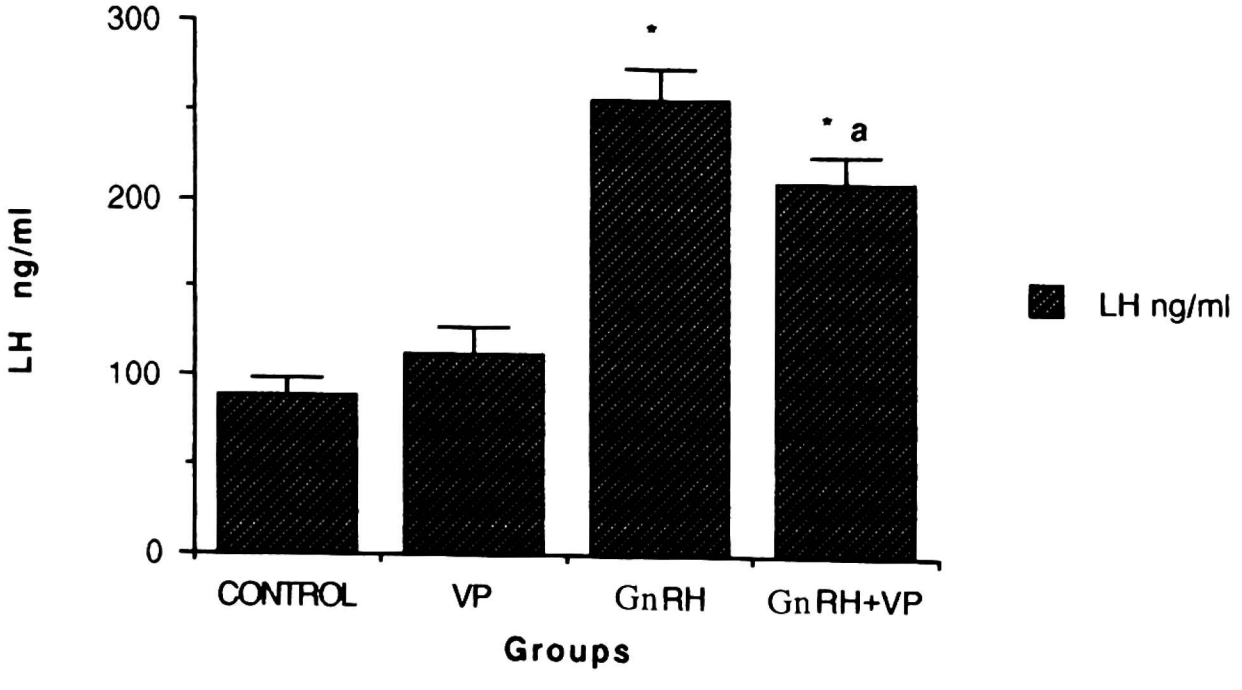

Fig. 8. Effect of verapamil (10 µg/ml) on the LH release in response to GnRH (20 ng/ml) by hemipituitaries from intact male rats. Number of samples per group: 8—10. * $p < 0.05$ vs Control or verapamil. [a] $p < 0.05$ vs GnRH.

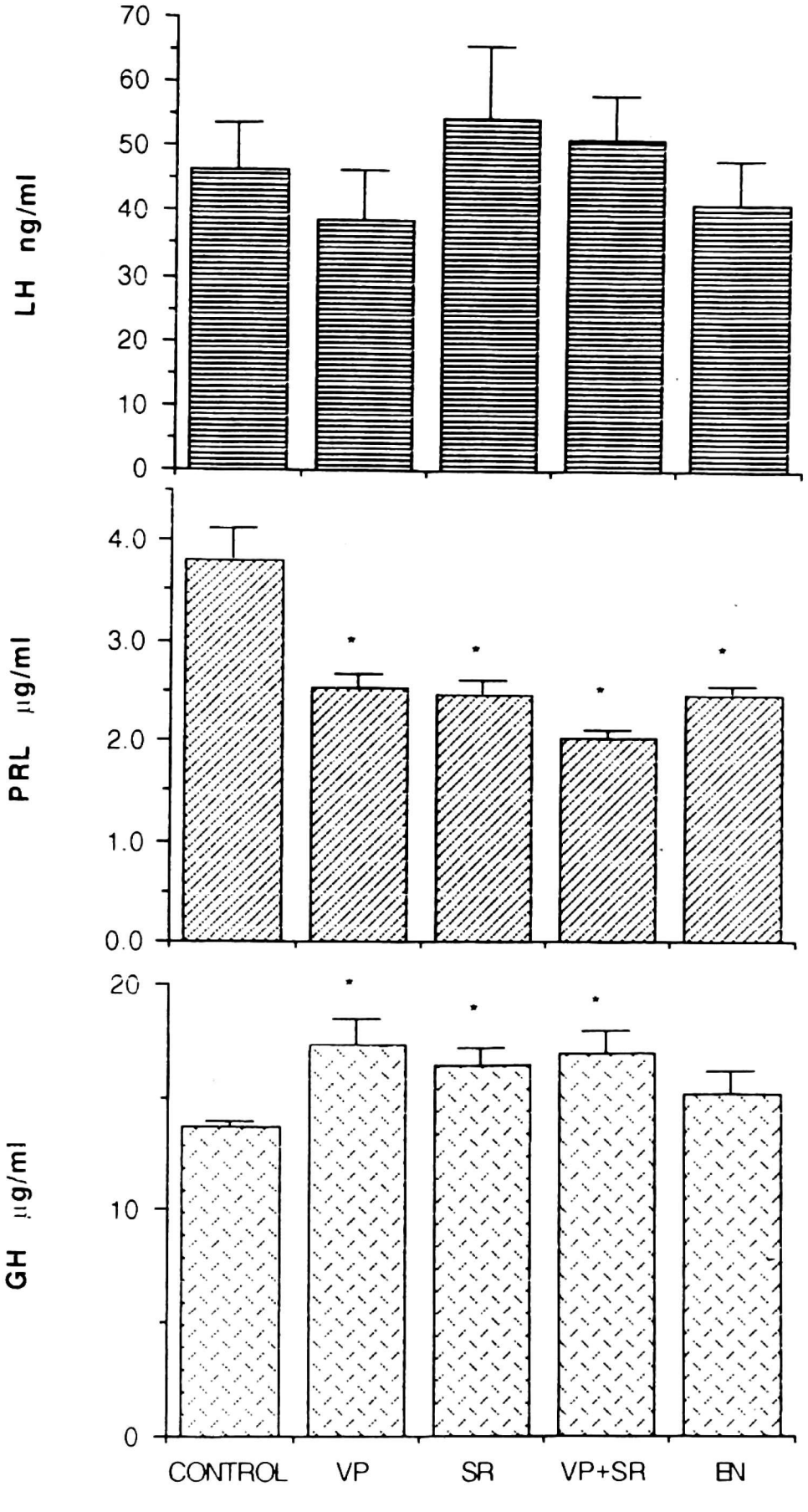

Fig. 9. Effect or verapamil, SR or both, or the (R)-enantiomer of SR (EN) on the release of LH (upper panel), PRL (middle panel), and GH (lower panel) by hemipituitaries from intact male rats. Number of samples per group: 10. * $p < 0.05$ vs Control.

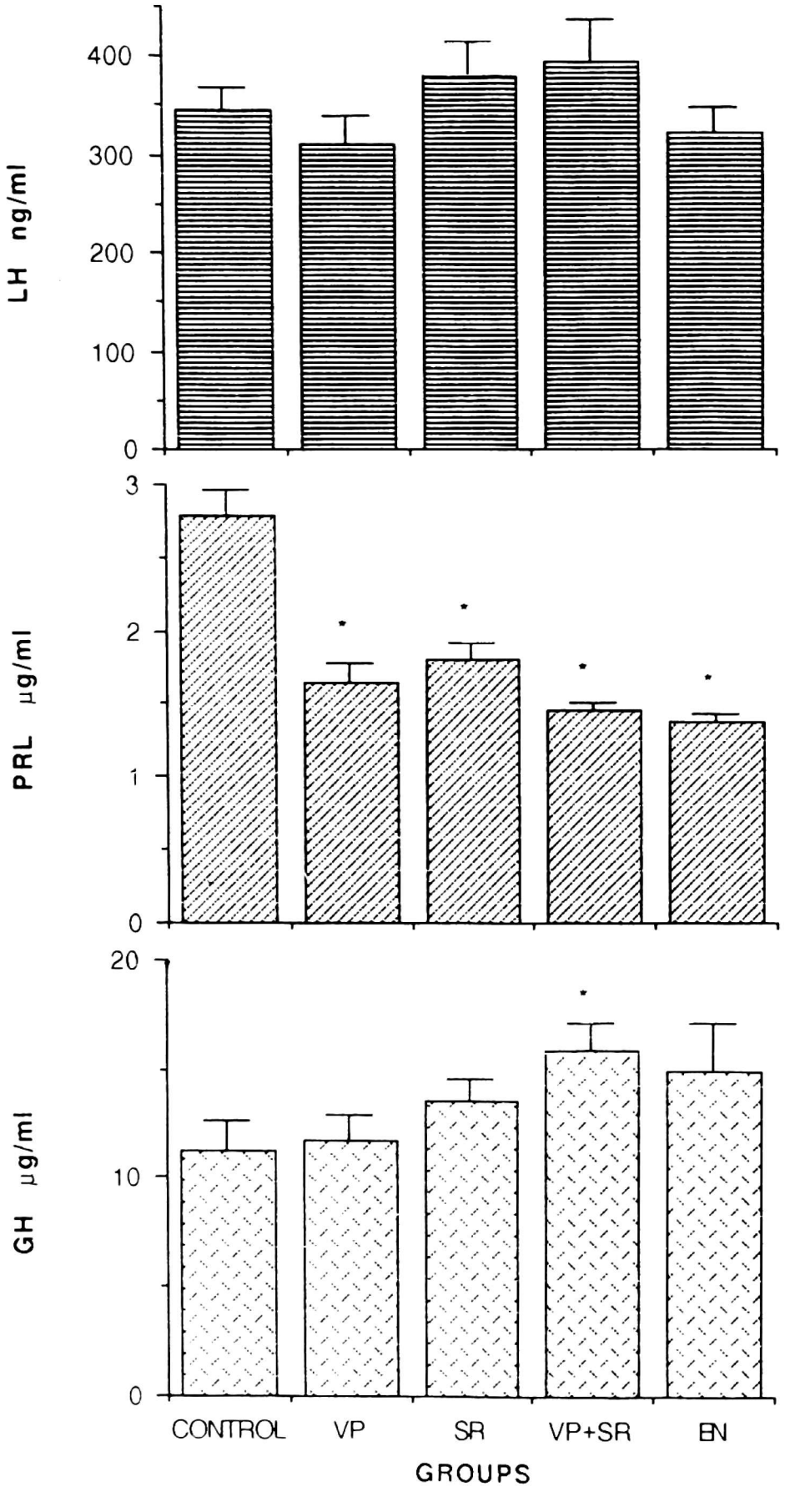

Fig. 10. Effect of verapamil, SR or both, or the (R)- enantiomer of SR (EN) on the release of LH (upper panel), PRL (middle panel), and GH (lower panel) by hemipituitaries from castrated male rats. Number of samples per group: 10. * $p < 0.05$ vs Control.

In the following incubation, using hemipituitaries from castrated male rats (*Fig. 10*), LH release was not significantly modified by any of the the substances tested, although the SR and SR plus verapamil groups had slightly higher LH release in the medium. As seen with hemipituitaries from intact rats, both verapamil and SR significantly inhibited PRL release into the medium ($p < 0.05$), the effect of the combination of both being more potent, although not reaching a statistically significant difference with SR or verapamil groups. EN also significantly decreased PRL release ($p < 0.05$). Regarding the release of GH, only verapamil and SR given together significantly stimulated GH release, the release of GH in the groups incubated with SR, verapamil, or EN not being significantly different from the control group.

EN did not significantly modify the release of LH in response to GnRH (Data not shown).

DISCUSSION

Although direct evidence on the existence of NK-2 receptors in the rat AP is still scant, the fact that the incubation of hemipituitaries with a potent NK-2 receptor antagonist in the present investigation resulted in significant effects suggests that these receptors may be present in the AP gland. The most consistent effect of the NK-2 receptor antagonist SR in our studies was the inhibition of PRL release. This effect of SR is in excellent agreement with our previous report that the intravenous injection of an antiserum to NKA resulted in decreased serum PRL levels (19). However, the control studies with verapamil and EN suggest the possibility that at least part of the effects of SR may be due to its Ca^{2+} channel antagonism. This is particularly evident in the case of PRL, in which both EN and verapamil were markedly active in decreasing the release of this hormone. Since EN was demonstrated to be markedly less active than SR on other systems (15), the fact that in our AP incubations it significantly affected PRL release, suggests that this effect was possibly mediated by Ca^{2+} channel antagonism. In the case of LH and GH however, EN was inactive, and verapamil increased GH release only from hemipituitaries of intact, but not castrated rats. EN was also inactive in modifying the response to GnRH.

The only case in which SR proved ineffective in modifying PRL release was when hemipituitaries were derived from ovariectomized rats that had been treated with estradiol (*Fig. 6*). In this incubation, the absolute levels of PRL were considerably higher than in the remaining incubations, presumably due to the *in vivo* effect of estradiol in stimulating PRL synthesis and release. It therefore appears that pituitaries exposed to

a chronic stimulatory effect of estradiol were unable to respond to the inhibitory effect of SR on PRL release.

Kalra *et al.* (8) reported that the incubation of hemipituitaries from intact male rats with NKA, NPK, or NPG resulted in an increase of LH release, and NPK and NPG also increased, although not significantly, the LH response to GnRH. Our finding that SR inhibited the response to GnRH is in agreement with the results described by Kalra *et al.* (8). Shamgochian and Leeman (10) found that SP also stimulated LH release by AP cells in culture. More difficult to explain, however, are the results obtained mainly with hemipituitaries from castrated and castrated testosterone-treated rats, in which a stimulation of the basal release of LH by the highest dose of SR was observed. Perhaps this is an effect evidenced in the AP from these animal models and not in AP from intact male rats, although there was some tendency toward and increased release of LH by SR in the first experiment (*Fig. 1*). It seems very likely therefore, that the levels of endogenous gonadal steroids could influence the response to these antagonists. This is particularly evident in the incubations with pituitaries from female rats, in which SR did not stimulate LH release by AP from ovariectomized rats but did significantly stimulate LH release by AP from ovariectomized, estradiol-treated rats. These results show that the *in vivo* administration of estradiol can markedly influence and modify the *in vitro* response to SR.

It had been previously shown that Ca^{2+} channel agonists stimulated PRL release *in vitro,* and their respective antagonists had the opposite effect (20). Furthermore, Ca^{2+} channel agonists were demonstrated to potentiate the LH release in response to GnRH (21), and this finding fits well with the decreased response to LH-RH incubated by the Ca^{2+} channel antagonist verapamil as observed in the present investigation. These results indicate that the interpretation of the effects of some NK-2 receptor antagonists, such as the one used in this investigation, should be made with great caution, since their action can partially be mediated through calcium channels. In our experiments verapamil induced effects on the release of PRL and on the LH response to LH-RH that were also very similar to those induced by SR. The similarity between the effects of SR and verapamil makes this possibility very likely. It must be pointed out, however, that the similarity of effects between SR and verapamil is not concluvise proof by itself that SR acts through both NK-2 receptors and Ca^{2+} channels. A recent report (22) suggested that a number of NK-1 receptor antagonists may exert some of their effects on PRL and GH release by mechanism not involving NK-1 receptors.

In summary, the results of this investigation show that non-peptide NK-2 antagonists, such as SR, are active in modifying the release of AP hormones *in vitro*. However, they should not be used as a tool to establish the physiological

role of tachykinins, since their effects may be the combination of actions exerted on NK-2 and Ca^{2+} channel receptors.

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