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Unexpected Effects of Peptide and Nonpeptide Substance P Receptor Antagonists on Basal Prolactin and Growth Hormone Release In Vitro

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HOUBEN, H. AND C. DENEF. *Unexpected effects of peptide and nonpeptide substance P receptor antagonists on basal prolactin and growth hormone release in vitro.* PEPTIDES **14**(1) 109–115, 1993.—The effect of peptide and nonpeptide substance P antagonists on prolactin (PRL) and growth hormone (GH) secretion was evaluated in three-dimensional rat anterior pituitary cell aggregates. [D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]]Substance P inhibited basal growth hormone (GH) release at a concentration range of 1–10 μM. At higher concentrations (50 μM), the analogue inhibited basal prolactin (PRL) release but provoked a tenfold stimulation of GH release. However, these latter two effects could neither be mimicked nor antagonized by the tachykinins substance P (10 μM), neurokinin A (10 μM), and neurokinin B (3.3 μM). The effects could also not be explained by agonism or antagonism at the level of other receptors (e.g., vasopressin, bombesin, angiotensin II, thyroid hormone-releasing hormone, vasoactive intestinal peptide, dopamine, adrenaline, acetylcholine). Remarkably the nonpeptide substance P antagonists R 30732 (10 μM), R 32602 (10 μM), and CP-96,345 (10 μM) showed a similar inhibition of PRL release and a stimulation of GH release. At a one hundredfold lower concentration, sufficient to block substance P receptors in other tissues, CP-96,345 did not affect PRL or GH release. It is concluded that substance P antagonists, when used at high concentrations, have profound intrinsic activities on PRL and GH release that are not mediated by substance P receptors. The failure of the more potent substance P antagonist, CP-96,345, to influence basal PRL or GH release when used at lower concentrations suggests that endogenous substance P in the anterior pituitary does not play a tonic paracrine role on GH or PRL secretion.

Antagonist Substance P Peptides Anterior pituitary

SEVERAL synthetic derivatives of substance P (SP), such as [D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]]substance P (SP-ant), have been shown to be competitive antagonists of SP (22,27). The peptides are, however, not very potent and are nonselective as they also block bombesin receptors (10,15,31), and antagonize the mitogenic action of vasopressin on 3T3 fibroblast cells (31) as well as a Ca^{++}-mobilizing action mediated by the receptor encoded by the *mas*-protooncogene (14), although the latter effect can be questioned in view of the recent findings from Ambroz et al. (1). These SP derivatives have also been reported to inhibit mitosis in small cell lung cancer cell lines (3,10,18,31), possibly by blocking autocrine growth stimulation by bombesin-like peptides produced by some of these cell lines.

As many biologically active peptides, among which are peptides of the tachykinin family, bombesin-like peptides, and vasopressin, are present in certain anterior pituitary cells (11,13,16), SP-ant could be used as a tool to explore the local role of these endogenous peptides. If the above-mentioned peptides are released by anterior pituitary cells, they might affect the secretion of various pituitary hormones such as prolactin (PRL) and growth hormone (GH) (12,21,29,30). In our search for a local role of peptides in the anterior pituitary, we thus looked to whether the addition of SP-ant, which blocks the receptors mediating the effects of several peptides, would provoke alterations in basal release of PRL and GH.

In the present paper we explore the effect of SP-ant, as well as of the nonpeptide SP antagonists R 30732, R 32602 (both from Janssen Pharmaceutica), and CP-96,345 (20), on basal PRL and GH release in rat anterior pituitary aggregate cell cultures.

METHOD

Peptides and Drugs

[D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]]Substance P (lot numbers 012085 and 015769), SP, neurokinin A (NKA), neuromedin C (NMC), rat growth hormone-releasing factor (GRF), and rat vasoactive intestinal peptide (VIP) were purchased either from Peninsula Laboratories Europe (Merseyside, UK) or from

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Bachem (Europe subsidiary, Hannover, Germany). Vasopressin, angiotensin II, and thyroid hormone-releasing hormone (TRH) were bought from UCB Bioproducts (Braine-l'Alleud, Belgium) and neurokinin B (NKB) from Sigma (Deisenhofen, Germany). R 30732, R 32602, and haloperidol were kind gifts from Dr. J. Leysen, Janssen Pharmaceutica (Beerse, Belgium). CP-96,345 {(2*S*,3*S*)-*cis*-2-(diphenylmethyl)-*N*-[(2-metoxyphenyl)-methyl]-1-azabicyclo-[2.2.2]octan-3-amine} was a gift from Dr. S. McLean (Pfizer Inc., Groton, USA). Propranolol HCl was obtained from Imperial Chemical Industries Ltd. (Pharmaceutical Division, Alderley Park), atropine sulfate from Merck AG (Darmstadt, Germany), L 686,095-001C002, the *N*-pivaloyl GRP(20–25) alkylamide $(CH_3)_3$-CCO-His-Trp-Ala-Val-Gly-His-NH-HC-$[(CH_2)_4CH_3]$ $[CH_2CH\ (CH_3)_2]$, was a gift from Dr. D. C. Heimbrook and Dr. W. S. Saari (Merck Sharp & Dohme Research Laboratories, West Point, PA). The peptides, propranolol, and atropine were dissolved in 0.9% NaCl containing 1% BSA and added 1:100 or 1:1000 during perifusion experiments, while R 30732, R 32602, and haloperidol were dissolved in ethanol and added 1:1000.

Isolation of the Cells and Preparation of the Aggregates

Male Wistar rats (9 to 12 weeks old) were killed by decapitation. Anterior pituitaries were dispersed into single cells that were suspended in serum-free defined culture medium as described previously (2,6,12). Culture medium was supplemented with 4 n*M* dexamethasone, 0.05 n*M* T3, and 0.8 mg/l phenolsulfonphthaleine. Cells were transferred into Petri dishes (Nunc, Roskilde, Denmark). By constant gyratory shaking at 65 rpm in a humidified CO_2-air incubator (1.2% CO_2) at 37°C, aggregates were obtained. These aggregates we used in a perifusion system on day 5 or 6 of culture. The ultrastructural integrity and functional specificity of such aggregates was shown previously (7).

Perifusion of Pituitary Cell Aggregates and RIA of Secreted PRL and GH

Perifusions were performed as previously described (2,7,12). Aggregates were transferred to a chamber of the perifusion system (1.6 to 2 × 10^6 cells/chamber) and allowed to adjust to the perifusion conditions for 150 min before the collection of perifusate started. The perifusion medium consisted of HEPES (25 m*M*)-buffered (pH 7.5), Dulbecco's modified Eagle's Medium (DMEM) supplemented with 1 g/l $NaHCO_3$ and 1 g/l NaCl. The flow rate was 0.25 ml/min. The perifusate was collected in 4-min fractions. Baseline secretion was measured for at least 28 min before secretagogues were administered as rectangular pulses. Pulses took at least 20 min, but when SP-ant was given together with other stimulatory or inhibitory products, SP-ant was given 12 min in advance in order to saturate receptors and was present 8 to 12 min longer than the other product. In control lines, SP-ant was added for the same period. All vehicles in which the test substances were dissolved were also present in the perifusion medium prior and subsequent to the administration of these substances. Prolactin and GH were measured in the perifusate using a specific rat PRL and GH RIA kit from Dr. A. F. Parlow (NIDDK, Baltimore, MD). The hormone–antibody complex was precipitated by *Staphylococcus aureus* membranes prepared in the laboratory of Dr. H. Eyssen (Department of Microbiology, K.U. Leuven). Reliability of the RIA was shown previously (2).

Statistics and Calculations

In order to determine whether observed changes in the hormonal secretion during pulses of agonists and antagonists are significant ($p < 0.05$), the amounts of GH or PRL released per 4 min in the fractions collected during the pulses were compared with the amounts in the baseline fractions before the pulse. For this comparison we used one-way analysis of variance (ANOVA) for single perifusion lines and two-way ANOVA (adapted to unequal sample groups) for multiple perifusion experiments (Number Cruncher Statistical System 5.1, graphics copyright Dr. J. L. Hintze, Kaysville). When there was a significant difference between basal release and release during the pulse, it is referred to as inhibition when the release decreases during the pulse, and stimulation when it increases. For evaluating the significance ($p < 0.05$) of rebound effects, we similarly compared the release in the fractions after the pulse with that in the baseline fractions.

As the number of cells in each perifusion chamber is not exactly identical, the release is expressed as a percentage of the basal release in order to be able to compare the effect in different perifusion lines. Thus, in figures showing the secretion pattern in perifusion lines (Figs. 1, 3–7) the percent of basal release for each point is the hormonal secretion per 4 min (duration of one perifusion fraction) expressed as a percentage of the mean basal release per 4 min as recorded in the period (usually 28 min) before the pulse.

In order to show the overall effect of a stimulus (e.g., fig. 2), the average hormonal secretion of all the fractions during the pulse (area under the curve) is presented as a percentage of the mean basal release as recorded in a comparable period of time before the pulse.

RESULTS

As shown in Fig. 1, perifusion of aggregates with 50 μM SP-ant provoked a rapid decrease of basal PRL release ($p < 0.001$, $n = 10$) but strongly stimulated GH release ($p < 0.001$, $n = 10$). Stimulation and inhibition were sustained over the period SP-ant was applied (40 min). Upon removal of SP-ant, secretion rapidly returned to baseline levels, although PRL inhibition was temporarily followed by a reversible rebound secretion ($p < 0.001$, $n = 10$) and GH stimulation by a fall below initial baseline levels ($p < 0.001$, $n = 10$). However, lower doses of SP-ant caused a decrease of basal GH release ($p < 0.001$ for 1, 5, and 10 μM, n indicated in Fig. 2). Inhibition of GH release was already seen at a dose of 1 μM ($p < 0.001$, $n = 2$) and was still present at a dose of 10 μM ($p < 0.001$, $n = 4$). At 1 and 5 μM

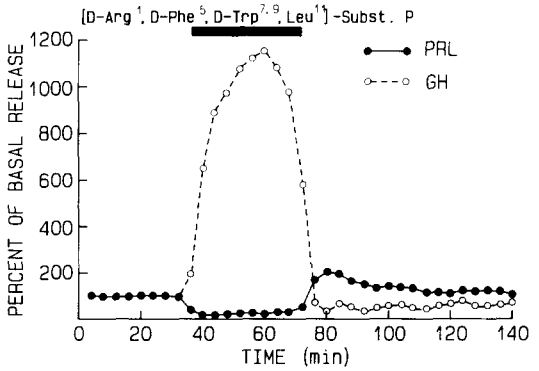

FIG. 1. Representative experiment showing the effect of 50 μM [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]SP on PRL and GH release in perifused reaggregate cell cultures of dispersed anterior pituitary cells from adult male rats. The bar indicates the exposure time to the stimulus.

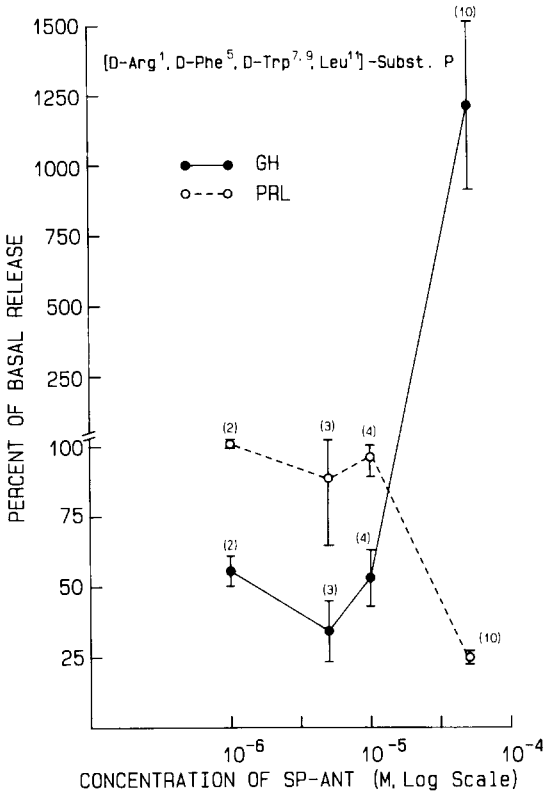

FIG. 2. Dose–response of the effect of [D-Arg¹,D-Phe⁵,D-Trp⁷·⁹,Leu¹¹]SP on PRL and GH release when administered for 40 min to perifused adult male rat anterior pituitary cell aggregates. Values (mean ± SEM of the number of experiments indicated in parentheses) represent the average GH or PRL release during exposure to the peptide as percent of the basal release (area under the curve).

no effect was seen on PRL release ($p < 0.05$), while at 10 μM two out of four experiments showed a significant inhibition of PRL release ($p < 0.05$). At 50 μM PRL release was inhibited to 25% of basal, whereas GH release was stimulated by a factor more than 10. Figure 2 shows the dose–response relationship of these SP-ant effects on GH and PRL release. In two independent cultures, not shown in Fig. 2, a 10 μM dose inhibited PRL release ($p < 0.001$) to the same level (21–27% of basal) as the 50 μM dose and this was associated with a ten- and twenty-threefold, respectively, rise in basal GH release ($p < 0.001$). Concentrations below 1 μM failed to affect PRL or GH release.

Two nonpeptide SP antagonists with receptor binding potency in the μM range, R 30723 and R 32602 ($IC_{50} \approx 10^{-5.85}$ and $10^{-6.08}$ M, respectively, in rat tissues; Dr. J. E. Leysen, unpublished data), showed comparable effects as SP-ant. The substances stimulated GH release ($p < 0.001$, $n = 2$) and inhibited PRL release ($p < 0.05$, $n = 2$) at a dose of 10 μM (Fig. 3). Upon removal of these agents, however, PRL release rather slowly returned to basal secretion levels. R 32602 tended to be somewhat more effective than R 30732. It should be noted that these products are nonselective, as they have some affinity for several receptors when used at μM concentrations (e.g., α_1-adrenergic, D_2-dopamine, serotonin $5HT_2$, Na^+ and Ca^{2+} channels; Dr. J. E. Leysen, unpublished data).

The new and more potent nonpeptide SP antagonist CP-96,345 (20) elicited comparable effects on GH and PRL release when used at 10 μM ($p < 0.001$, $n = 3$). At 1 μM GH release was still slightly enhanced ($p < 0.001$, $n = 3$) and PRL release inhibited ($p < 0.001$, $n = 3$), but CP-96,345 had no effect when used at lower concentrations ($p \geq 0.05$, $n = 3$ for 0.1 and 0.01 μM (Fig. 4). The inhibition of PRL release by CP-96,345 returned to baseline secretion more rapidly after removal of the antagonist than the inhibition by R 30723 or R 32602 (Figs. 3 and 4).

The effects of SP-ant (50 μM) on PRL and GH release could not be antagonized by the tachykinin agonists SP (0.01 and 10 μM), NKA (10 μM), and NKB (3.3 μM) (Fig. 5). Substance P (10 μM) also failed to overcome the effect of R 30723, R 32602, and CP-96,345 on PRL and GH release (data not shown). Other agonists, like the bombesin-like peptide NMC (0.1 and 1 μM), vasopressin (1 μM), and angiotensin II (1 or 10 μM), which were previously shown to be affected by SP-ant in other tissues (10,14,15,31), were also unable to overcome the effect of 50 μM SP-ant on PRL and/or GH release (data not shown). Thyroid hormone-releasing hormone (1 μM) and VIP (0.5 μM), peptides well known to stimulate PRL release (5,19), failed to overcome the inhibitory action of SP-ant on PRL release, and although TRH inhibits GH release when added to our test system (Fig. 6) (8), TRH (1 μM) was unable to affect the GH release by SP-ant.

The magnitude and pattern of stimulation of GH release as a function of time by epinephrine (2), the bombesin-like peptide NMC (12), vasopressin, and VIP (2,8) markedly differed from that of SP-ant (Fig. 6), while TRH (1 μM) decreased GH release

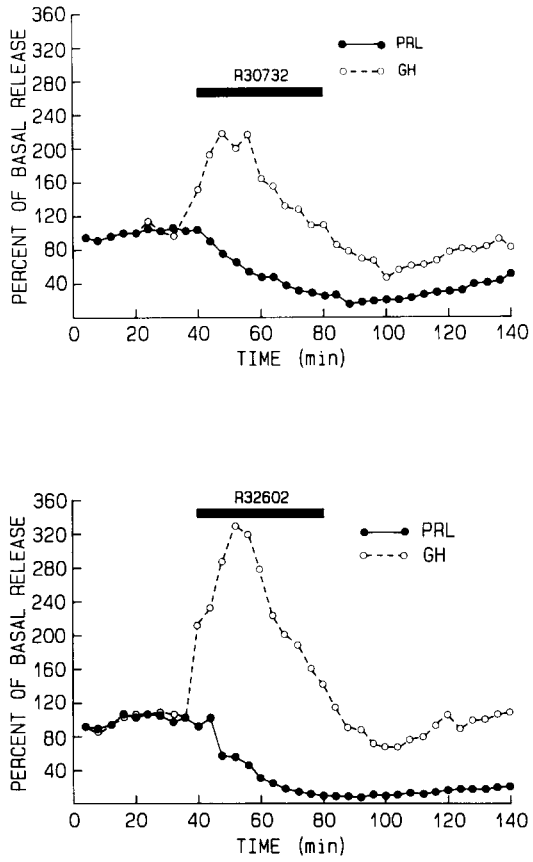

FIG. 3. Representative experiment showing the effect of 10 μM R 30732 and R 32602 on GH and PRL release in perifused reaggregate cell cultures of dispersed anterior pituitary cells from adult male rats. The bar indicates the exposure time to the stimulus.

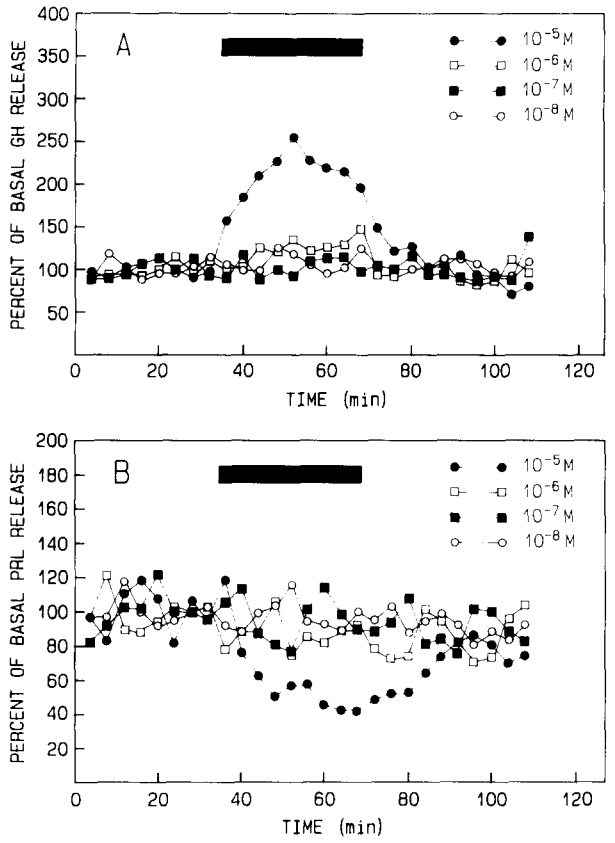

FIG. 4. Representative experiment showing the effect of different concentrations of CP-96,345 on GH (A) and PRL (B) release in perifused reaggregate cell cultures of dispersed anterior pituitary cells from adult male rats. The bar indicates the exposure time to the stimulus.

($p < 0.001$) in the condition where SP-ant stimulates GH release. Substance P (0.01 and 10 μM), NKA (10 μM), and NKB (10 μM) did not mimic SP-ant on GH or PRL release, as they had only a marginal stimulatory effect on the release of these hormones ($p < 0.05$ in all cases, Fig. 7). Vasopressin (1 μM) did not mimic the action of SP-ant either, as it provoked a 162% rise of PRL release and had no or only a marginal stimulatory effect on GH release ($n = 1$, Fig. 6). Neuromedin C stimulated GH and PRL release as reported elsewhere (12), while angiotensin II, opposite to the SP-ant, stimulates PRL release and inhibits GH release (in adult rat pituitary cells) (23–25). Thus, none of these peptides mimicked SP-Ant. Moreover, β-receptors, muscarinic receptors, bombesin or dopamine receptors are not involved, as the β-adrenoceptor antagonist propranolol (0.1 μM), the muscarinic receptor blocker atropine (0.1 μM), and the potent bombesin receptor blocker *N*-pivaloyl-GRP(20–25) alkylamide (L 686,095-001C002) (2 nM) (9) did not affect the stimulation of GH release by SP-ant (50 μM) (data not shown). Inhibition of PRL release by SP-ant (50 μM) was not blocked by the dopamine receptor antagonist haloperidol (0.1 μM), the muscarinic receptor blocker atropine (0.1 μM), or the bombesin receptor blocker L 686,095-001C002 (2 nM) (data not shown).

The only stimulus that can be compared with SP-ant as far as magnitude and pattern are concerned is GRF (Fig. 6, compare with Fig. 1). However, GH secretion decreased below baseline secretion shortly after a pulse of SP-ant 50 μM (Fig. 1), while it remained at a level higher than baseline secretion after a GRF pulse (0.1 nM) (Fig. 6).

DISCUSSION

In the present investigation, profound effects of the SP derivative [D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]]substance P (SP-ant) were found on basal PRL and GH release from perifused rat anterior pituitary cell aggregates. The effect on PRL release is strongly inhibitory, whereas that on GH release is dual: inhibition at a lower concentration range (1–10 μM) and a strong stimulation at higher concentrations (50 μM). These effects were observed at concentrations comparable to those reported previously for SP-ant to block SP (27,31), bombesin (31), and vasopressin (31) receptors in other tissues. Nonpeptide SP antagonists had similar effects.

The observation that effects on GH and PRL release occur with several types of SP antagonists suggested to us that it is a typical aspect of SP antagonism, and that the observed effects were due to antagonism of actions of endogenous SP on PRL and GH release. As the tachykinins SP, NKA, and NKB could not overcome the effect of SP-ant, this theory cannot be supported. The effects of SP-ant on PRL and GH release also cannot be due to antagonism towards endogenous pituitary bombesin-like peptides, vasopressin, or angiotensin II, which were previously shown to be affected by SP-ant in other tissues (10,14,15,31), since these peptides did not override the effect of SP-ant. The possibility that SP-ant, having a wide antagonist spectrum in the anterior pituitary, creates its effects by antagonizing other peptides like TRH and VIP has to be ruled out as well, since these peptides fail to overcome the effects of SP-ant. Thus, although the aim of this study was to use these SP-antagonists to find evidence for a local tonic action of one of the above-mentioned peptides, the data indicate that the effects of the SP antagonists are not due to counteracting a putative tonic local (paracrine) influence of tachykinins, bombesin-like peptides, vasopressin, angiotensin II, VIP, or TRH on hormone secretion.

We examined the possibility that SP-ant has some partial agonist action at the receptors for which it has known affinity in other tissues (e.g., tachykinin, bombesin, vasopressin, angiotensin II, *mas*-oncogene receptor) or at other receptors by using known receptor antagonists and by comparing the effect of SP-ant to that of various agonist stimuli. However, the antagonists did not affect the GH and PRL responses to SP-ant and none of the tested agonist stimuli mimicked the pattern of those responses. We thus found no evidence that the effects of SP-ant on GH and PRL release are due to a partial agonist action on the receptors of the above-mentioned peptides, nor on β-adrenergic, muscarinic, or dopamine receptors.

Recently, SP-ant was shown to compete with the synthetic GH-releasing peptide (GHRP) His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2 for binding on its receptors in the pituitary (28). An effect on this receptor may be responsible for the inhibitory effect of low concentrations of SP-ant on GH release, as SP-ant behaves as a competitive antagonist of the GH-releasing action of GHRP when added together (4) and, thus, may antagonize an endogenous peptide acting at the GHRP receptor. It also remains possible that SP-ant reaches its effect by simultaneously interfering with several (peptide) systems, thus profoundly disturbing the microenvironment of the anterior pituitary cells.

It is remarkable that three totally different classes of SP antagonists (e.g., the SP derivatives, the nonpeptide SP antagonists from Janssen, and those from Pfizer) exhibit comparable effects

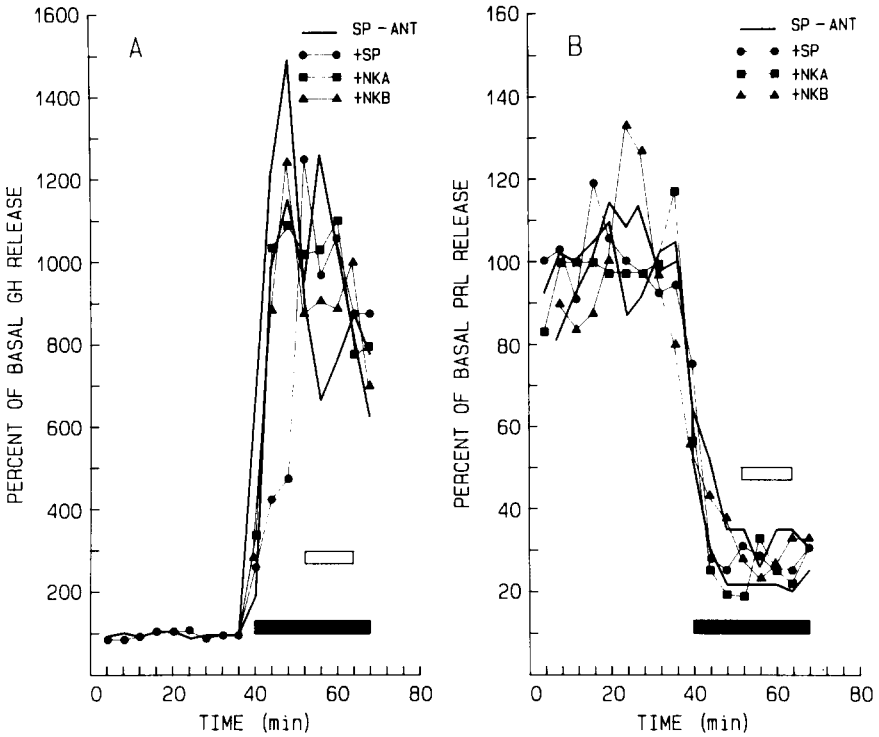

FIG. 5. Representative experiments showing the lack of effect of SP (10 μM), NKA (10 μM), and NKB (3.3 μM) on the GH (A) and PRL (B) secretory response to SP-ant (50 μM). Two perifusion lines in which only SP-ant was added are drawn in the darker solid line. The SP-ant (exposure time indicated by solid bar) was added 12 min before the additional stimulus with SP, NKA, or NKB (exposure time indicated by open bar).

on GH and PRL release when used at high concentrations. This suggests a common site of action, with binding requirements related to those of the SP receptor. Although this common site remains unknown, it is possible that the effects of SP-ant, as well as the nonpeptide antagonists, are the consequence of an interaction with the dihydroxypyridine (DHP) receptor, particularly because they were seen at relatively high concentrations of the compounds. An interaction of SP-ant with DHP binding sites of voltage-dependent Ca^{2+} channels could even explain the opposite effects on GH and PRL release. Indeed, it is known that DHPs (such as nifidepine) block these Ca^{2+} channels, while other DHPs act as agonists, and still others [such as (+) 202791 and Bay K 8644] enhance Ca^{2+} currents in hyperpolarized cells whereas they block Ca^{2+} currents in depolarized cells (16). If the SP antagonists behave as DHP agonists in somatotrophs but as blocker of Ca^{2+} channels in lactotrophs, this would explain the stimulation of GH release and the inhibition of PRL release. The latter hypothesis is supported by receptor binding data of the nonpeptide SP antagonists R 30732 and R 32602. These substances have μM affinity for DHP binding sites (IC_{50} $10^{-6.4}$ and $10^{-6.15}$ M, respectively; Dr. J. E. Leysen, personal communication of unpublished data). Also, the related peptide [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]SP (10 μM) was previously shown to inhibit the effects of angiotensins on intracellular Ca^{2+} exerted via the *mas*-oncogene receptor in transfected oocytes and in a transfected neural cell line (14). This finding strengthens our suggestion that the SP antagonists may generally affect Ca^{2+} currents.

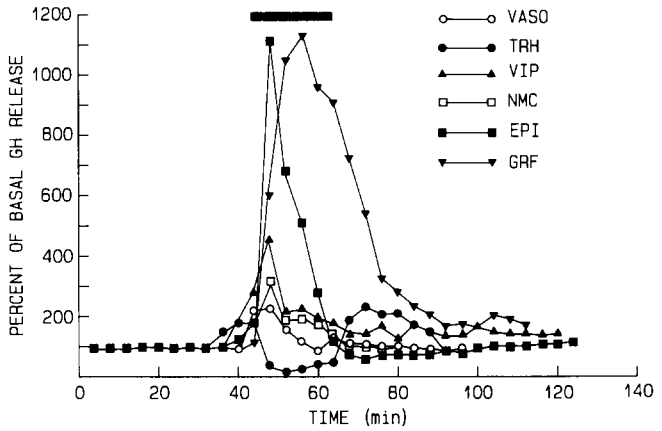

FIG. 6. Representative experiments (in different cell cultures) showing the GH stimulation pattern as a function of time for GRF (10^{-10} M), epinephrine (Epi, 3×10^{-8} M), neuromedin C (NMC, 10^{-7} M), vasoactive intestinal peptide (VIP, 5×10^{-7} M), vasopressin (Vaso, 10^{-6} M), TRH (10^{-6} M) when added in separate perifusion lines to reaggregate cell cultures of dispersed anterior pituitary cells from adult male rats. The bar indicates the exposure time to the stimuli.

The new nonpeptide SP antagonist CP-96,345 also enhanced GH release and inhibited PRL release when used at high concentrations. For this antagonist, however, the SP antagonizing concentrations reported for other tissues are at least 10 to 100 times lower than the concentrations eliciting these GH and PRL responses (20). CP-96,345 thus seems to be a more selec-

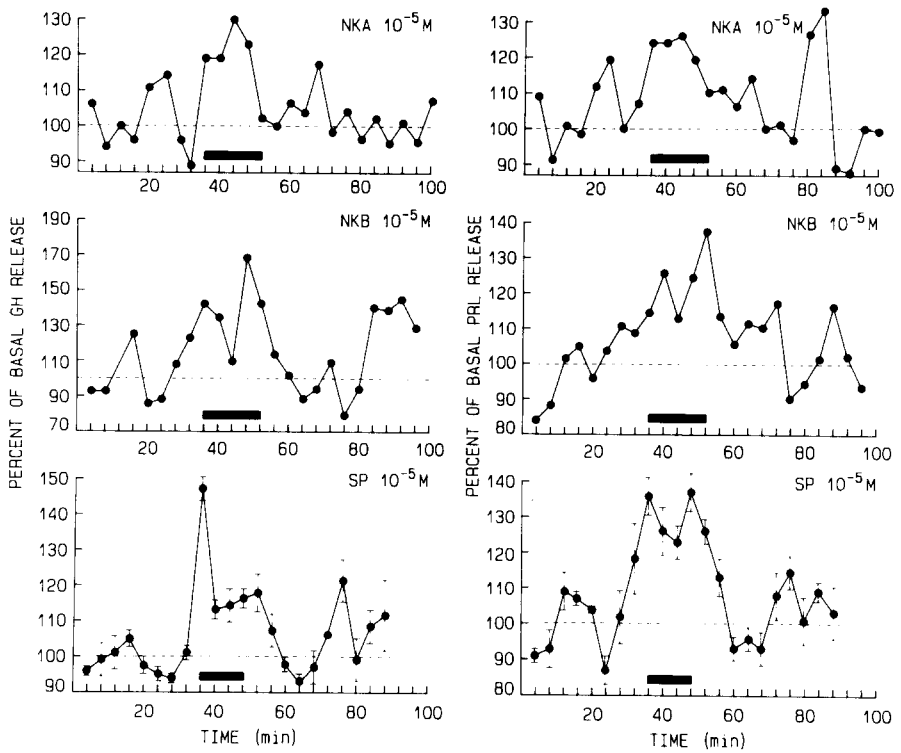

FIG. 7. Stimulation of GH and PRL release by NKA (10 μM), NKB (10 μM), and SP (10 μM). For NKA and NKB results from a single experiment are presented while for SP data represent mean (±SEM) of three independent perifusion experiments. The bar indicates the exposure time to each stimulus.

tive SP antagonist. Since CP-96,345 had no effect on PRL and GH release at these lower concentrations, we suggest that endogenous SP has no tonic paracrine action on PRL or GH secretion in the pituitary, at least not at the NK_1 receptor subtype (20), which is the main type of SP receptor in the anterior pituitary (17).

In conclusion, the present investigation shows peculiar secretory effects of peptide and nonpeptide SP antagonist on PRL and GH release that do not seem to be due to agonist or antagonist actions at a single known receptor except perhaps the DHP receptor. No evidence in favor of a local paracrine action of endogenous pituitary SP could be obtained.

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