Antagonistic actions of analogs related to growth hormone-releasing hormone (GHRH) on receptors for GHRH and vasoactive intestinal peptide on rat pituitary and pineal cells *in vitro*

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Peptide analogs of growth hormone-releasing hormone (GHRH) can potentially interact with vasoactive intestinal peptide (VIP) receptors ($VPAC_1$-R and $VPAC_2$-R) because of the structural similarities of these two hormones and their receptors. We synthesized four new analogs related to GHRH (JV-1–50, JV-1–51, JV-1–52, and JV-1–53) with decreased GHRH antagonistic activity and increased VIP antagonistic potency. To characterize various peptide analogs for their antagonistic activity on receptors for GHRH and VIP, we developed assay systems based on superfusion of rat pituitary and pineal cells. Receptor-binding affinities of peptides to the membranes of these cells were also evaluated by radioligand competition assays. Previously reported GHRH antagonists JV-1–36, JV-1–38, and JV-1–42 proved to be selective for GHRH receptors, because they did not influence VIP-stimulated $VPAC_2$ receptor-dependent prolactin release from pituitary cells or $VPAC_1$ receptor-dependent cAMP efflux from pinealocytes but strongly inhibited GHRH-stimulated growth hormone (GH) release. Analogs JV-1–50, JV-1–51, and JV-1–52 showed various degrees of $VPAC_1$-R and $VPAC_2$-R antagonistic potency, although also preserving a substantial GHRH antagonistic effect. Analog JV-1–53 proved to be a highly potent $VPAC_1$ and $VPAC_2$ receptor antagonist, devoid of inhibitory effects on GHRH-evoked GH release. The antagonistic activity of these peptide analogs on processes mediated by receptors for GHRH and VIP was consistent with the binding affinity. The analogs with antagonistic effects on different types of receptors expressed on tumor cells could be utilized for the development of new approaches to treatment of various human cancers.

growth hormone-releasing hormone and vasoactive intestinal peptide antagonists | structure–activity relationships | cancer therapy

Growth hormone-releasing hormone (GHRH) is a member of a superfamily of structurally related peptide hormones that includes vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP), secretin, and glucagon (1). Receptors for these peptides belong to a family of seven-transmembrane-spanning G protein-coupled receptors (2). GHRH exerts its action through high-affinity GHRH receptors (GHRH-R) predominantly present in the anterior pituitary (3, 4). VIP binds to two subtypes of VIP receptors (VPAC-R), previously called VIP_1 and VIP_2 receptors or PACAP type 2 receptors, because they also have a high affinity for PACAP, and therefore were recently named $VPAC_1$ and $VPAC_2$ receptors ($VPAC_1$-R and $VPAC_2$-R) (5). These receptors with different tissue distribution and pharmacological properties are distinct from the specific highly selective PACAP type 1 receptors (PAC_1-R) that recognize VIP with a low affinity (6). These peptides bind with high affinity to their respective receptors and in addition are also able to crossreact in various degrees, in general with reduced affinity, with the receptors of the other members of this superfamily because of the structural similarity of the peptides and their receptors (7).

Native GHRH has a low affinity to VPAC-R, whereas its synthetic derivatives have various affinities to these binding sites (7–10). The first reported human GHRH (hGHRH) antagonist [Ac-Tyr^1, D-Arg^2]hGHRH(1–29)NH_2 proved to be a weak VPAC-R agonist when tested on rat pancreatic membranes (8–10). In contrast, another analog of GHRH [Ac-Tyr^1, D-Phe^2]hGHRH(1–29)NH_2 had a pronounced VPAC-R inhibitory activity (8, 10), and it also exerted a partial GHRH agonistic effect (9, 10).

Antagonistic analogs of GHRH have been synthesized in many laboratories (9, 11–16) because of their expected applications (17–21). These analogs could be useful for therapy of endocrine disorders such as acromegaly, diabetic retinopathy, or diabetic nephropathy. However, the main applications of GHRH antagonists would be in the field of cancer (17, 19–21). GHRH antagonists synthesized in this laboratory (14–16) inhibit tumor growth in experimental animals acting: (*i*) indirectly through pituitary GHRH-R leading to the suppression of GHRH-GH-insulin-like growth factor (IGF)-I axis; or (*ii*) directly via the reduction of IGF-I and IGF-II production in tumors (21). GHRH antagonists also inhibit the proliferation of various cancers *in vitro* apparently by a direct action on cancer cells (22, 23). Because the classic pituitary type GHRH-R are not present on tumor cells, but VPAC-R are abundant in many malignancies (23–25), GHRH antagonists can potentially interact with these VPAC-R and inhibit tumor proliferation. To investigate these interactions, we synthesized four new antagonistic peptide analogs based on the structure of GHRH but designed to have reduced effect on GHRH-R and increased activity on VPAC-R. For the simultaneous characterization of these peptide analogs for their antagonistic activity on GHRH-R, $VPAC_1$-R, and $VPAC_2$-R, we developed an *in vitro* dynamic biological assay based on superfusion systems. The advantages and benefits of the dispersed cell superfusion system, as applied to GHRH antagonists, were reported earlier in comparison with assays in static cultures (26). In our dispersed cell superfusion system, the tissue culture medium is perfused continuously, thus reducing the occurrence of local hormonal feedbacks. The test materials

Abbreviations: AP, anterior pituitary; GH, growth hormone; GHRH, GH-releasing hormone; GHRH-R, GHRH receptor; hGHRH, human GHRH; NET INT, net integral value; PACAP, pituitary adenylate cyclase-activating polypeptide; PG, pineal gland; PRL, prolactin; VIP, vasoactive intestinal peptide; VPAC-R, VIP/PACAP receptor.

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can be applied in a more physiological pulsatile fashion, and the dynamics of hormone response and the changes in the responsiveness can also be analyzed.

According to the earlier pharmacological and molecular studies on VPAC-R, only $VPAC_2$-R has been identified in the pituitary gland (27), whereas in the pineal gland, $VPAC_1$-R seemed to be dominant (28). Consequently, we used dispersed pituitary cells for testing the antagonistic activities of our newly synthesized analogs on GHRH-R and $VPAC_2$-R and dispersed pineal cells for studying their inhibitory effects on $VPAC_1$-R. Antagonistic activities of these compounds on GHRH-R and $VPAC_2$-R were evaluated by the inhibition of GHRH-stimulated growth hormone (GH) release and VIP-induced prolactin (PRL) secretion, respectively. Inhibitory potency of these peptides on $VPAC_1$-R was determined by the blockade of VIP-evoked cAMP efflux from pinealocytes. The effects of these analogs were compared with those produced by a highly selective $VPAC_1$-R antagonist (PG 97–269) (29) and our recently reported potent GHRH antagonists JV-1–36, JV-1–38, and JV-1–42 (16).

This paper describes the characterization of GHRH-related peptide analogs by using the superfusion method and radioligand competition assay for evaluating their inhibitory potencies on GHRH-R, $VPAC_1$-R, and $VPAC_2$-R.

Materials and Methods

Peptides. The synthesis of hGHRH(1–29)NH_2, and GHRH analogs JV-1–36, JV-1–38, and JV-1–42 was previously described (16). Analogs JV-1–50, JV-1–51, JV-1–52, and JV-1–53 were synthesized, purified, and analyzed by the same methods (16). Briefly, manual solid-phase peptide synthesis by using *tert*-butyloxycarbonyl-protected amino acids was carried out on *para*-methylbenzhydrylamine resin followed by hydrogen fluoride cleavage of the finished peptides. Crude products were purified by semipreparative HPLC and checked by analytical HPLC and amino acid analyses (16). VIP was obtained from California Peptide Research (Napa, CA). Potent $VPAC_1$-R selective antagonist (PG 97–269) was kindly provided by P. Gourlet and P. Robberecht (Université Libre de Bruxelles, Belgium) (29).

Superfusion. The superfusion of dispersed pituitary cells was performed as described earlier (26, 30). This system also applied for pinealocytes with some modifications. Briefly, for each experiment, anterior pituitaries (AP) and pineal glands (PG) of two young adult male Sprague–Dawley rats were digested with 0.75% collagenase CLS 2 (Worthington) for 50 min for AP and 20 min for PG. After incubation, the fragments were dispersed into clusters (5–40 cells) by mechanical dispersion, then transferred onto two columns for AP or one column for PG and allowed to sediment simultaneously with 0.8 ml Sephadex-G (Sigma). Medium 199 (Sigma) containing BSA (1 g/liter), $NaHCO_3$ (2.2 g/liter), penicillin G (50 mg/liter) (Sigma), and gentamicin sulfate (87 mg/liter) (Sigma) was equilibrated with a mixture of 95% air/5% CO_2 and used as the culture medium. After an overnight recovery period, the cells regained their full responsiveness. First, the system was standardized with 3-min exposures to 1 nM hGHRH(1–29)NH_2 or 10 nM VIP for AP and with 6-min exposure to 10 nM VIP for PG. The antagonists were infused at various concentrations for 9 min. This was immediately followed by the mixture of an antagonist and 1 nM GHRH or 10 nM VIP for an additional 3 min for AP and an antagonist and 10 nM VIP for 6 min in the case of PG. The duration of the antagonistic effect was checked by the subsequent infusions of 1 nM GHRH or 10 nM VIP at 30-min intervals for AP and 60-min intervals for PG. Each experiment was performed in three superfusion columns (GHRH-R, $VPAC_1$-R, and $VPAC_2$-R antagonist test) simultaneously. Immediately after collection of fractions (1 ml/3 min), 25 μl of freshly prepared mixture of triethylamine and acetic anhydride (2:1 vol/vol) was added to 500 μl ice-cold aliquots of the medium fractions for RIA of cAMP. These aliquots were kept frozen at −20°C together with the rest of the collected fractions for RIA of growth hormone (GH) and PRL.

Receptor Binding. The preparation of rat anterior pituitary and pineal membrane fractions and receptor binding of GHRH and VIP were performed as reported (31, 32). Sensitive *in vitro* ligand competition assays based on the binding of radiolabeled [His^1,Nle^{27}]hGHRH(1–32)NH_2 and radiolabeled VIP to rat anterior pituitary and pineal membrane homogenates were used. A radioiodinated derivative of [His^1,Nle^{27}]hGHRH(1–32)NH_2 was prepared as described (31), and ^{125}I-labeled VIP was purchased from Amersham. In brief, membrane homogenates containing 30–80 μg protein were incubated at 24°C in duplicate or triplicate with 50–80,000 cpm radioligand and increasing concentrations (10^{-12}–10^{-6} M) of nonradioactive peptides as competitors (31, 32). Receptor-binding affinities were calculated by the LIGAND-PC computerized curve fitting program of Munson and Rodbard, as modified by McPherson (33).

RIA. The levels of rat GH and PRL in collected medium as well as cAMP levels in aliquots of acetylated medium were determined by double-antibody RIA. The antibodies (anti-rat GH-RIA-5/AFP-411S, anti-rat PRL-S-9/AFP-131581570, anti-cAMP-NIDDK CV-27), the reference preparations (rat GH-RP-2/AFP-3190B, rat PRL-RP-3/AFP-4459B), and the hormones for iodination (rat GH-I-6/AFP-5676B, rat PRL-I-6/AFP-10505B) were provided by A. F. Parlow (Harbor-UCLA Medical Center, Torrance, CA), whereas the standard cAMP and tyrosyl-methyl ester-cAMP for iodination were purchased from Sigma.

Mathematical Analysis. The results of RIA were analyzed with a computer program developed in our institute (30) involving ANOVA and Student's *t* test. The net integral values (NET INT) of responses exposed to drugs were calculated (expressed as mean ± SEM) and compared. The NET INT is the difference between the total area under the peak and the area under the baseline along the peak representing the net amount of hormones and nucleotide released in response to stimulus.

Results

Peptide Synthesis. In an attempt to produce GHRH analogs with increased VPAC-R antagonistic activities and decreased GHRH-R antagonistic properties, four peptides (JV-1–50, JV-1–51, JV-1–52, and JV-1–53), derived from the sequence of hGHRH(1–29)NH_2, were prepared by solid-phase synthesis (Table 1). After purification, the purity of peptides was found to be >95%. Amino acid analyses of the pure products showed the expected amino acid compositions.

Receptor-Binding Affinities. Binding assays for GHRH-R and VPAC-R were performed on rat anterior pituitary and pineal tissue preparations by using two radioligands [^{125}I][His^1,Nle^{27}]hGHRH(1–32)NH_2 and [^{125}I]VIP (Table 2). JV-1–36 and JV-1–38 displayed the highest binding affinity to rat pituitary GHRH-R, but their affinity to pituitary and pineal VPAC-R was at least 100-fold weaker than that of VIP.

The binding affinity to GHRH-R of peptides such as JV-1–50, JV-1–51, and JV-1–52, designed to have partly VIP antagonistic properties, was weaker than that of hGHRH(1–29)NH_2. This affinity was also two orders of magnitude lower than that of GHRH antagonists JV-1–36 and JV-1–38. Two of these analogs, JV-1–51 and JV-1–52, displayed relatively high affinity binding to pituitary VPAC-R.

Table 1. Comparative structures of hGHRH(1–29)NH_2, GHRH analogs, selective $VPAC_1$-R antagonist PG 97-269, and VIP

Amino acid residue		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	
hGHRH (1-29)NH_2	H-	Tyr	Ala	Asp	Ala	Ile	Phe	Thr	Asn	Ser	Tyr	Arg	Lys	Val	Leu	Gly	Gln	Leu	Ser	Ala	Arg	Lys	Leu	Leu	Gln	Asp	Ile	Met	Ser	Arg	-NH_2
JV-1-36	PhAc-	·	D-Arg	·	·	·	Phe(4-Cl)	·	·	Arg	·	·	·	·	·	Abu	·	·	·	·	·	·	·	·	·	·	·	Nle	D-Arg	Har	-NH_2
JV-1-42	PhAc-	His	D-Arg	·	·	·	Phe(4-Cl)	·	·	Arg	·	·	·	·	·	Abu	·	·	·	·	·	·	·	·	·	·	·	Nle	D-Arg	Har	-NH_2
JV-1-50	PhAc-	His	D-Phe	·	·	·	Phe(4-Cl)	·	·	Arg	·	·	·	·	·	Abu	·	·	·	·	·	·	·	·	·	·	·	Nle	D-Arg	Har	-NH_2
JV-1-51	Ac-	His	D-Phe	·	·	·	Phe(4-Cl)	·	·	Arg	·	·	·	·	·	Abu	·	·	·	·	·	·	·	·	·	·	·	Nle	D-Arg	Har	-NH_2
JV-1-38	PhAc-	·	D-Arg	·	·	·	Phe(4-Cl)	·	·	Har	Tyr(Me)	·	·	·	·	Abu	·	·	·	·	·	·	·	·	·	·	·	Nle	D-Arg	Har	-NH_2
JV-1-52	Ac-	His	D-Phe	·	·	·	Phe(4-Cl)	·	·	Har	Tyr(Me)	·	·	·	·	Abu	·	·	·	·	·	·	·	·	·	·	·	Nle	D-Arg	Har	-NH_2
JV-1-53	Ac-	His	D-Phe	·	·	·	Phe(4-Cl)	·	·	·	·	·	·	·	·	Lys	Arg	·	·	·	Lys	·	Tyr	·	·	·	·	Nle	D-Arg	Har	-NH_2
PG 97-269	Ac-	His	D-Phe	·	·	Val	·	·	·	·	·	·	·	·	·	Lys	Arg	·	·	·	·	·	·	·	·	·	·	Leu	-NH_2		
VIP	H-	His	Ser	·	·	Val	·	·	Asp	Asn	·	Thr	Arg	Leu	Arg	Lys	·	Met	Ala	Val	Lys	·	Tyr	·	Asn	Ser	·	Leu	Asn	-NH_2	

Amino acid residues identical to those of hGHRH(1-29)NH_2 are denoted by dots.

JV-1–53, designed to be an exclusive VIP antagonist, had an almost negligible affinity for GHRH-R, similar to that of VIP and the selective $VPAC_1$-R antagonist PG 97–269. In contrast, JV-1–53 had the highest binding affinity, even higher than VIP itself, to VPAC-R on pituitary and pineal cells. The selective $VPAC_1$-R antagonist (PG 97–269) exhibited high affinity binding to pineal VPAC-R but showed very weak binding to pituitary VPAC-R.

Effect of GHRH Analogs and a Selective $VPAC_1$-R Antagonist on GHRH-Stimulated GH Response. Inhibitory effects of these peptides on GHRH receptors were evaluated further in a dispersed rat pituitary superfusion system. Pulsatile stimulation of GH cells with 1 nM hGHRH(1–29)NH_2 for 3 min at 30-min intervals caused a sharp increase in GH secretion (Fig. 1), whereas it did not influence the basal PRL secretion (data not shown). The GH release quickly reached the maximum value in 3–6 min and then rapidly returned to basal levels. The areas under the peaks (NET INT) were equivalent to 892.3 ± 28.1 ng GH, except for the first GH response in which the NET INT was higher (1,606.0 ng). Because this high first GH response was a general phenomenon in all experiments, the NET INT of the second GH response was used as reference value in the subsequent inhibitory tests. In these tests, the cells were first preincubated with antagonistic analogs at 10- to 100-nM concentrations for 9 min and then immediately exposed to a mixture of the analogs and 1 nM GHRH for an additional 3 min (Fig. 1). The duration of the inhibitory effect of these analogs on the responsiveness of GH cells was evaluated by the infusion of 1 nM GHRH 30, 60, and 90 min later. According to the results obtained from the superfusion system (Table 3), the order of potencies of these analogs was: JV-1–36 = JV-1–42 > JV-1–38 ≫ JV-1–52 >

Table 2. Relative binding affinities of GHRH-related peptides to membrane receptors for GHRH and VIP on rat anterior pituitary and pineal cells

Peptide	Relative affinity to pituitary GHRH-R*	Relative affinity to pituitary VPAC-R†	Relative affinity to pineal VPAC-R‡
hGHRH(1-29)NH_2	1	<0.001	N/A
VIP	<0.001	1	1
JV-1-36	79	<0.01	N/A
JV-1-38	42	<0.01	<0.001
JV-1-50	0.2	<0.01	N/A
JV-1-51	0.08	0.4	N/A
JV-1-52	0.2	0.8	N/A
JV-1-53	<0.001	1.1	1.9
PG 97-269	<0.001	<0.001	0.9

Binding affinities were determined by using a nonlinear curve fitting program (33) for analysis of ligand competition studies, as described. Values represent mean of duplicate determinations. N/A, data not available.

*Expressed relative to the binding affinity of hGHRH(1-29)NH_2 to rat pituitary GHRH-R (K_i = 3.34 nM).

†Expressed relative to the binding affinity of VIP to rat pituitary VPAC-R (K_i = 1.13 nM).

‡Expressed relative to the binding affinity of VIP to rat pineal VPAC-R (K_i = 16.4 nM).

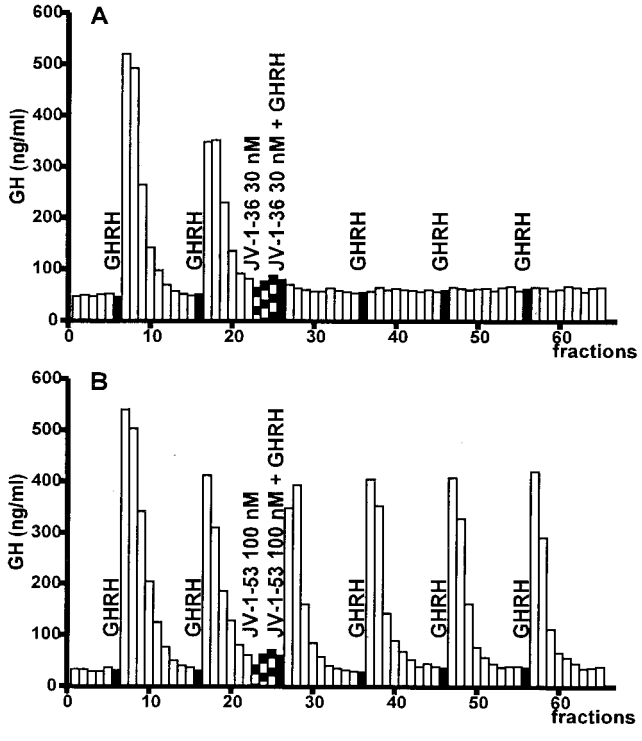

Fig. 1. The effects of JV-1–36 (30 nM) (*A*) and JV-1–53 (100 nM) (*B*) on basal and GHRH-induced GH release from rat pituitary cells in a superfusion system. After two 3-min infusions of 1 nM GHRH (solid filled bars), the cells were exposed to JV-1–36 or JV-1–53 for 9 min (checkered bars), followed by a simultaneous infusion of these analogs and 1 nM GHRH for an additional 3 min (filled bar) (0-min inhibition). The duration of the inhibitory effect of these analogs was evaluated by three consecutive 3-min infusions of 1 nM GHRH at 30-min intervals (filled bars) (30-, 60-, and 90-min inhibition). The second GHRH-induced GH response, before the antagonist exposure was used as a reference value for calculations.

Table 3. Inhibitory effects of peptide analogs on the hGHRH(1-29)NH_2-induced GH release in rat pituitary cell superfusion system

Peptide		Inhibition of GH release, %*			
Code	Dose	0 min	30 min	60 min	90 min
JV-1-36	10 nM	57	59	61	56
	30 nM†	100	100	100	100
JV-1-38	10 nM	46	53	51	54
	30 nM†	85	98	91	92
JV-1-42	10 nM	62	64	43	34
	30 nM†	97	91	82	76
JV-1-50	30 nM	38	34	25	11
JV-1-51	30 nM	37	0	0	2
JV-1-52	30 nM	72	5	4	4
JV-1-53	30 nM	0	0	0	4
	100 nM	7	4	9	20
PG 97-269	30 nM	5	0	8	17
	100 nM	2	10	9	5

*Calculated from NET INT of GH responses after the second infusion of 1 nM GHRH (NET $INT_{GHRH} = 1.0$) and after simultaneous infusion of 1 nM GHRH and antagonist (NET $INT_{GHRH+ANT}$) as $100 \times (1.0\text{-NET } INT_{GHRH+ANT})/1.0$.
†From ref. 16.

Table 4. Inhibitory effects of peptide analogs on the VIP-induced cAMP efflux in rat pineal cell superfusion system

Peptide		Inhibition of cAMP efflux, %*	
Code	Dose	0 min	60 min
JV-1-36	300 nM	4	0
	1,000 nM	58	24
JV-1-38	300 nM	9	46
	1,000 nM	22	56
JV-1-42	300 nM	18	10
	1,000 nM	65	4
JV-1-50	300 nM	57	0
	1,000 nM	76	28
JV-1-51	30 nM	55	13
	100 nM	83	20
JV-1-52	300 nM	59	12
	1,000 nM	92	57
JV-1-53	30 nM	13	30
	100 nM	100	10
PG 97-269	30 nM	38	29
	100 nM	73	36

*Calculated from NET INT of cAMP responses after the second infusion of 10 nM VIP (NET $INT_{VIP} = 1.0$) and after simultaneous infusion of 10 nM VIP and antagonist (NET $INT_{VIP+ANT}$) as $100 \times (1.0\text{-NET } INT_{VIP+ANT})/1.0$.

JV-1–50 > JV-1–51 ≫ JV-1–53 ≅ PG 97–269, which is consistent with their GHRH receptor-binding affinities (Table 2). JV-1–36 (Fig. 1*A*), JV-1–38, and JV-1–42, designed as GHRH antagonists containing D-Arg^2 in their peptide sequence, caused a particularly strong and long-lasting inhibition of responsiveness of GH cells. In contrast, those analogs with D-Phe^2 substitution, which had been designed to possess primarily VIP antagonistic characteristics (JV-1–50, JV-1–51, JV-1–52, and JV-1–53), proved to be much weaker GHRH antagonists, with the exception of JV-1–52, which had a relatively strong but brief inhibitory effect. JV-1–53 (Fig. 1*B*) and the selective $VPAC_1$-R antagonist (PG 97–269) did not inhibit GHRH stimulated GH response at all, even at 100 nM concentration.

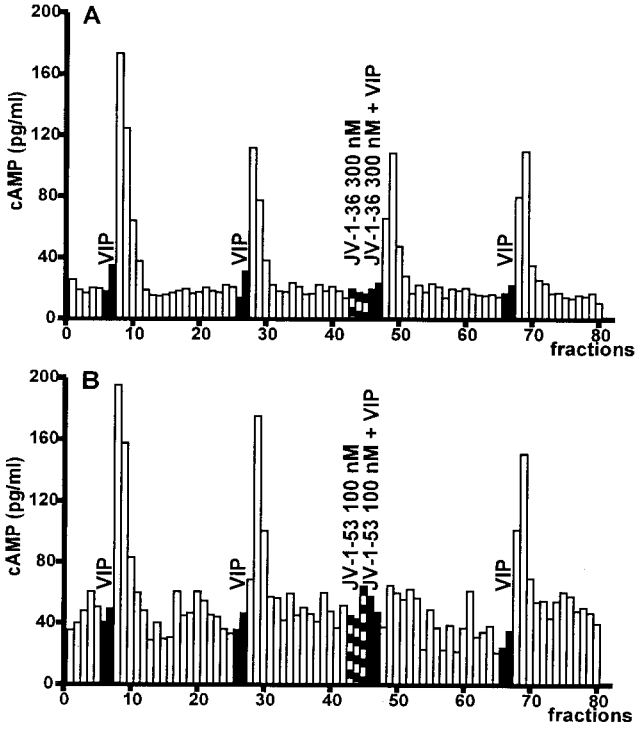

Fig. 2. The effects of JV-1–36 (300 nM) (*A*) and JV-1–53 (100 nM) (*B*) on basal and VIP-induced cAMP efflux from rat pineal cells in a superfusion system. After two 6-min infusions of 10 nM VIP (solid filled bars), the cells were exposed to JV-1–36 or JV-1–53 for 9 min (checkered bars) followed by a simultaneous infusion of these analogs and 10 nM VIP for an additional 6 min (filled bars) (0-min inhibition). The duration of the inhibitory effect of these analogs was evaluated by a 6-min infusion of 10 nM VIP in 60 min (filled bars) (60-min inhibition). The second VIP-induced cAMP response, before the antagonist exposure was used as reference value.

Effect of GHRH Analogs and a Selective $VPAC_1$-R Antagonist on VIP-Stimulated cAMP Efflux from Pinealocytes. Antagonistic activity of these analogs on $VPAC_1$-R was evaluated in the dispersed rat pinealocyte superfusion system. VIP (10 nM) infused alone for 6 min at 60-min intervals evoked a prompt increase in cAMP efflux from pinealocytes (Fig. 2). The release of cyclic nucleotide started to increase immediately after the exposure to VIP, reaching the maximal value in the first 6–9 min, and then declined to the basal values. NET INT of the first VIP-induced cAMP response was higher than the others during the experiment (676.2 ng vs. 361.9 ± 3.78 ng), and consequently the second VIP-stimulated cAMP response was used as the reference peak in these experiments. In this test, an antagonistic analog was infused for 9 min at various concentrations (30 nM to 1,000 nM), which was immediately followed by the simultaneous infusion of the analog and 10 nM VIP for 6 min (Fig. 2). The duration of the antagonistic effect was checked 60 min later with a single infusion of 10 nM VIP. The inhibitory potencies of these analogs on $VPAC_1$-R in the superfusion system (Table 4) were similar to those obtained from radioligand competition assay (Table 2) and proved to be essentially the opposite of their GHRH antagonistic activities, their order being: JV-1–53 ≅ JV-1–51 ≅ PG 97–269 ≫ JV-1–52 > JV-1–50 > JV-1–42 ≅ JV-1–36 ≅ JV-1–38. JV-1–53 (Fig. 2*B*) and JV-1–51 at 100 nM concentration strongly inhibited the effect of VIP, in a manner similar to the selective $VPAC_1$-R antagonist PG 97–269. Among the other analogs, JV-1–50 and JV-1–52 inhibited VIP-stimulated cAMP efflux when administered at higher (300 nM) concentration, whereas JV-1–36 (Fig. 2*A*), JV-1–38, and JV-1–42 proved to be ineffective at 300 nM concentration.

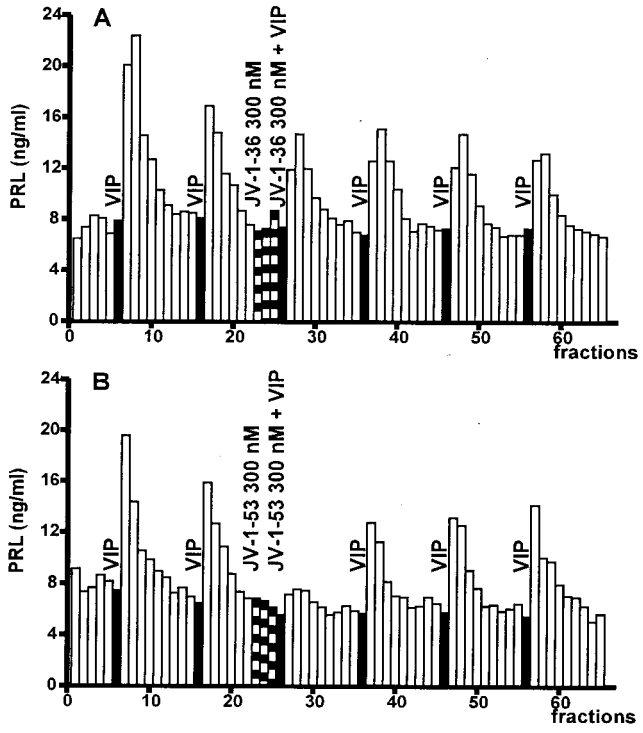

Fig. 3. The effects of JV-1–36 (300 nM) (*A*) and JV-1–53 (300 nM) (*B*) on basal and VIP-induced PRL release from rat pituitary cells in a superfusion system. After two 3-min infusions of 10 nM VIP (solid filled bars), the cells were exposed to JV-1–36 or JV-1–53 for 9 min (checkered bars) followed by a simultaneous infusion of these analogs and 10 nM VIP for an additional 3 min (filled bar) (0-min inhibition). The duration of the inhibitory effect of these analogs was evaluated by three consecutive 3-min infusions of 10 nM VIP at 30-min intervals (filled bars) (30-, 60-, and 90-min inhibition). The second VIP-induced PRL response before the antagonist exposure was used as reference value.

Effect of GHRH Analogs and a Selective $VPAC_1$-R Antagonist on VIP-Stimulated PRL Response. Antagonistic activities of these analogs on $VPAC_2$-R were also tested in the dispersed rat pituitary superfusion system. VIP itself at 10 nM concentration was able to stimulate PRL secretion and when infused for 3 min at 30-min intervals (Fig. 3), it rapidly elevated PRL release, which reached a peak in 3–6 min and then returned to basal values. NET INT of the first PRL release was 40.4 ng, approximately 2-fold higher than that of subsequent responses, and consequently the second PRL response, evoked by VIP, was used as reference. The administration of antagonistic analogs at 300 nM concentration for 9 min was followed by the infusion of a mixture of an antagonist and VIP (10 nM) for 3 min (Fig. 3). To check the duration of the antagonistic effect, 10 nM VIP was applied 30, 60, and 90 min later for 3 min. The order of potencies in this inhibitory test was as follows (Table 5): JV-1–53 > JV-1–52 > JV-1–51 ≫ JV-1–42 ≅ PG 97–269 ≅ JV-1–50 ≅ JV-1–38 ≅ JV-1–36, being in agreement with the results from the receptor-binding assay (Table 2). Among the D-Phe^2 containing analogs, designed to behave as antagonists of VIP, JV-1–51, JV-1–52, and JV-1–53 had variable $VPAC_2$-R antagonistic activity, JV-1–53 (Fig. 3*B*) being the most potent. In contrast, both the selective $VPAC_1$-R antagonist PG 97–269 and our D-Arg^2- containing GHRH antagonists, JV-1–36 (Fig. 3*A*), JV-1–38, and JV-1–42, were practically ineffective on VIP-stimulated PRL release at concentrations tested. The D-Phe^2- containing analog JV-1–50 also lacked measurable inhibitory effect on PRL release.

Discussion

For the *in vitro* characterization of various peptide analogs based on the structure of GHRH, we established and used two different dispersed cell superfusion systems modifying earlier methods (26, 34). Regarding the localization of VPAC-R, only $VPAC_2$-R was identified in the pituitary gland (27), whereas in the pineal gland, $VPAC_1$-R appears to play a role in the activation of VIP-evoked melatonin secretion (28). Therefore, the activity of our analogs on GHRH-R and $VPAC_2$-R was evaluated in a dispersed pituitary superfusion system, whereas their inhibitory effect on $VPAC_1$-R was simultaneously studied on dispersed pinealocytes. Using this combination of dynamic *in vitro* systems, we could obtain information about the structure–activity relationships of the peptide analogs in comparison with the results from *in vitro* ligand competition assay.

Table 5. Inhibitory effects of peptide analogs on the VIP-induced PRL release in the rat pituitary cell superfusion system

Peptide		Inhibition of PRL release, %*			
Code	Dose	0 min	30 min	60 min	90 min
JV-1-36	300 nM	8	0	13	27
JV-1-38	300 nM	14	0	5	21
JV-1-42	300 nM	24	3	7	13
JV-1-50	300 nM	15	17	6	6
JV-1-51	300 nM	42	6	18	21
JV-1-52	300 nM	69	28	43	36
JV-1-53	300 nM	100	47	24	18
PG 97-269	300 nM	16	12	21	22

*Calculated from NET INT of PRL responses, as indicated in Table 4 legend.

Antagonistic analogs of GHRH JV-1–36, JV-1–38, and JV-1–42 (Table 1) were previously synthesized in our laboratory as part of our program to develop highly potent and long-acting GHRH antagonists for potential therapeutic use. These peptides contain the D-Arg^2 substitution that is known to produce predominantly GHRH antagonistic property when incorporated into the analogs of GHRH (9, 10). Thus JV-1–36, JV-1–38, and JV-1–42 proved to be selective GHRH-R antagonists, because they bound to GHRH-R with high affinity and blocked the GH-releasing effect of GHRH in the pituitary cell superfusion system but were ineffective to inhibit $VPAC_1$-R and $VPAC_2$-R.

Analogs JV-1–50, JV-1–51, JV-1–52, and JV-1–53 (Table 1) were intended to be VIP antagonists and contained the D-Phe^2 substituent instead of D-Arg^2, because this substitution was reported to produce predominantly VIP antagonistic property on incorporation into GHRH analogs (8, 10). The structures of VIP antagonists JV-1–50 and JV-1–51 are closely related to those of GHRH antagonist JV-1–36 and JV-1–42, the only differences between these four compounds being in the first two amino acids and the *N*-acyl moiety. The structure of VIP antagonist JV-1–52 is the most closely related to the structure of GHRH antagonist JV-1–38, differing from it only in the Ac-His^1-D-Phe^2 sequence. Thus, the modification of the structures of these peptides by replacing D-Arg^2 by D-Phe^2 resulted in analogs JV-1–50, JV-1–51, and JV-1–52 with substantially decreased GHRH inhibitory potency and GHRH-R binding affinity, but having significant $VPAC_1$-R and variable $VPAC_2$-R antagonistic activity. The most potent $VPAC_1$-R antagonist among these three analogs was JV-1–51, which showed an inhibitory activity similar to that of specific $VPAC_1$-R antagonist PG 97–269. In addition, analog JV-1–50 had weak, whereas JV-1–51 and JV-1–52 had stronger, $VPAC_2$-R antagonistic activity.

VIP antagonist JV-1–53 has several additional substitutions as compared with JV-1–50, JV-1–51, and JV-1–52, intended to increase its binding to both $VPAC_1$-R and $VPAC_2$-R and decrease its affinity to the GHRH-R. On the basis of the report of Gourlet *et al.* (29), the incorporation of Lys^{15} (also found in native VIP) and Arg^{16} residues in this peptide was expected to

increase $VPAC_1$-R antagonistic potency, because their potent and selective $VPAC_1$-R antagonist PG 97–269 contains these substitutions (29). Tyr^{22} was expected to increase the binding to $VPAC_2$ receptors and confer enhanced $VPAC_2$-R antagonistic activity to the analog, based on the published observations regarding the importance of an aromatic amino acid residue in position 22 for $VPAC_2$-R agonists (35). In agreement with this assumption, JV-1–53 had a strong antagonistic effect on $VPAC_2$ receptors in addition to its potent $VPAC_1$-R inhibitory activity. To our knowledge, JV-1–53 could be the most potent $VPAC_2$-R antagonist reported so far. JV-1–53 also contains Lys^{20} substitution, which is characteristic of native VIP. The replacement of Arg ^{20} by Lys^{20} in GHRH analogs was reported to drastically reduce their GH-releasing activities (36), and consequently we believed that this replacement in JV-1–53 would result in decreased affinity to GHRH receptors. Our results support this hypothesis, because JV-1–53 was not able to inhibit GHRH-stimulated GH response, and its GHRH-R-binding affinity decreased by more than four orders of magnitude compared with GHRH antagonists JV-1–36 and JV-1–38.

The selective $VPAC_1$-R antagonist PG 97–269 caused a strong inhibition in VIP-evoked cAMP efflux from dispersed pinealocytes. This is consistent with the data reported earlier, that in the rat pineal gland VIP acts on $VPAC_1$-R (28) to stimulate melatonin secretion through cAMP production and that this effect can be blocked by a VIP antagonist (37). In the pituitary superfusion, the selective $VPAC_1$-R antagonist PG 97–269 did not significantly influence the basal or VIP-induced PRL release. These findings can be explained by the fact that in PRL cells, in addition to three variants of mRNA for PAC_1-R, only $VPAC_2$-R mRNA, but not $VPAC_1$-R mRNA is present (27). In our dispersed pituitary superfusion system, the selective $VPAC_1$-R antagonist PG 97–269 was similarly not able to block the GHRH-stimulated GH response. The results of receptor-binding assays support these findings, because PG 97–269 had a very low affinity to both GHRH-R and VPAC-R on pituitary cells.

In conclusion, this report describes the characterization by sensitive *in vitro* assay systems of various peptide analogs related to the structure of GHRH with respect to their inhibitory potencies on GHRH-R, $VPAC_1$-R, and $VPAC_2$-R. The compounds tested ranged from primarily GHRH antagonists, comprising JV-1–36, JV-1–38, and JV-1–42 to selective VIP antagonist (JV-1–53) and included nonselective analogs acting on both GHRH-R and VPAC-R, such as JV-1–50, JV-1–51, and JV-1–52. Consequently, it is expected that from studies with these analogs in various cancer models, useful findings can be obtained on the types of receptors involved in the antiproliferative mechanism.

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