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# Study of Species Specificity in Growth Hormone-Releasing Factor (GRF) Interaction with Vasoactive Intestinal Peptide (VIP) Receptors Using GRF and Intestinal VIP Receptors from Rat and Human: Evidence that Ac-Tyr[1]hGRF is a Competitive VIP Antagonist in the Rat

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## SUMMARY

In order to determine species specificity in growth hormone-releasing factor (GRF) interaction with vasoactive intestinal polypeptide (VIP) receptors, we have tested rat (r) GRF (with a His[1] such as in VIP), human (h) GRF and position 1 substituted analogs of hGRF (Ala[1], Ac-Tyr[1], His[1], Phe[1], and Trp[1] in the place of Tyr[1]) for their ability to inhibit $^{125}$I-VIP binding and to stimulate adenylate cyclase activity in human and rat intestinal epithelial membranes. We show that rGRF has a much higher affinity than hGRF for both human and rat VIP receptors. In humans, the $K_i$ values for inhibiting $^{125}$I-VIP binding are 0.5 (VIP), 26 (rGRF), and 830 nM (hGRF). In rats the values are 0.6 (VIP), 46 (rGRF), and 1100 nM (hGRF). This is due in part to the presence of His[1] in rGRF since the analog His[1] hGRF has a higher affinity than hGRF in man and rat, i.e., $K_i$ = 320 nM and 460 nM, respectively. Studies of adenylate cyclase stimulation reveal that rGRF and His[1]hGRF are full VIP agonists in man and rat, whereas hGRF and its other analogs behave as partial agonists in both species. One of the hGRF analogs tested (Ac-Tyr[1]hGRF) is of great interest since it inhibits $^{125}$I-VIP binding to rat intestinal membranes with a $K_i$ = 430 nM but has a negligible intrinsic activity in stimulating adenylate cyclase activity (about 6% of the efficacy of VIP). This analog does inhibit the VIP-stimulated adenylate cyclase activity in a dose-dependent manner, complete inhibition of the VIP (0.01–1 nM) effect being obtained with 30 μM analog. The Schild plot of the inhibitory effect further indicates competitive antagonism. In contrast, Ac-Tyr[1]hGRF is a partial VIP agonist in humans (about 20% of the efficacy of VIP). These results evidence the important role of His[1] for peptide interaction with VIP receptors and provide the first example of a competitive VIP antagonist.

VIP (1) is a member of a rapidly growing family of peptides that now includes secretin (2), glucagon (3), GIP (4), PHI (5) and its human counterpart, PHM (6, 7), and GRF (8, 9). VIP triggers biological responses through specific binding to cell surface receptors which are efficiently coupled to adenylate cyclase in most target tissues (10). VIP receptors have been studied extensively in rat (r) and human (h) intestinal epithelium (11) where their structural requirements were investigated using natural (12, 13) and synthetic (14) analogs of VIP. It appeared that secretin (15, 16), PHI/PHM (12, 13), and hGRF (12) but not glucagon and GIP (15, 16) were able to interact with VIP receptors, although with a lower affinity than VIP. Important species specificity was observed, including a different ability of human and rat VIP receptors to recognize natural VIP-related peptides (12, 13) as well as partial VIP sequences (14), indicating that VIP receptors have evolved in man toward a high degree of specificity of the recognition site (13).

In this connection, the isolation of rGRF (17) and the synthesis of position 1 substituted analogs of hGRF (18) were of great interest. rGRF has an N-terminal histidine such as in VIP instead of a tyrosine in hGRF. This is an important feature, especially as the N-terminal histidine in VIP was shown to be important not only for the recognition by VIP binding sites (14) but also for generating the biological response, e.g., the stimulation of adenylate cyclase activity (14, 19). In particular, VIP 2-28 (14), hGRF (12), and position 1 substituted analogs of secretin (16) and VIP (19) behave as partial VIP agonists in the rat.

This paper deals with the ability of rGRF, hGRF, and position 1 substituted analogs of hGRF, including his[1]hGRF, to compete with $^{125}$I-VIP binding and to stimulate adenylate cyclase activity in rat and human intestinal epithelial plasma membranes. We show that rGRF has a much higher affinity

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**ABBREVIATIONS:** VIP, vasoactive intestinal peptide; GIP, gastric inhibitory peptide; PHI, porcine peptide histidine isoleucine amide; PHM, peptide histidine methionine amide; GRF, growth hormone-releasing factor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

than hGRF for VIP receptors in both species, a feature related in part to its N-terminal histidine. We also demonstrate that the analog Ac-Tyr$^1$hGRF behaves as a competitive VIP antagonist in the rat, providing the first example of an antagonist for this neuropeptide.

## Materials and Methods

**Peptides and chemicals.** Porcine VIP was obtained from Prof. V. Mutt (Karolinska Institute, Stockholm, Sweden). rGRF, hGRF-44-$NH_2$, and position 1 substituted analogs of hGRF-40-OH (Table 1) as Ala$^1$, Phe$^1$, His$^1$, Trp$^1$, and Ac-Tyr$^1$hGRF-40-OH were synthesized by the solid phase method (18, 20) and were obtained from Dr. N. Ling (The Salk Institute for Biological Studies, La Jolla, CA). $^{125}$I-VIP (750 Ci/mmol) was prepared as described (21). It displays the same activity as native VIP in stimulating cyclic AMP accumulation in a cultured cell line (HT-29) which is highly and specifically sensitive to VIP (22). Synthetic ATP, cyclic AMP, GTP, 3-isobutyl-1-methylxanthine, phosphocreatine kinase, and bacitracin were obtained from Sigma Chemical Co. (St. Louis, MO). Other chemicals were of highest purity commercially available.

**Plasma membrane preparations.** Fresh specimens of human colon were obtained during segmental resection for cancer. In each case a 10-cm-long fragment was cut from the healthy upper end of the surgical piece. Isolation of epithelial crypts was performed using ethylenediaminetetraacetate as described (23). Small intestinal epithelial cells were prepared as described (15) from 2-month-old male Wistar rats. Plasma membranes were prepared from isolated cells as reported elsewhere in detail (24). Membrane proteins were determined by the method of Bradford (25) with bovine serum serum albumin as standard.

**Binding of $^{125}$I-VIP to membranes.** Plasma membranes (about 50 $\mu$g of protein/ml) were incubated at equilibrium as described (26) for 30 min at 30° with $^{125}$I-VIP (0.05 nM) and, when necessary, other compounds in 250 $\mu$l of 60 mM HEPES buffer, pH 7.5, containing 2% (w/v) bovine serum albumin and 0.1% (w/v) bacitracin. The reaction was stopped by transferring a 200-$\mu$l aliquot portion of the incubation medium onto 200 $\mu$l of ice-cold 60 mM HEPES buffer, followed by immediate centrifugation for 10 min at 30,000 × $g$. The resulting pellet was washed two times with 400 $\mu$l of ice-cold 60 mM HEPES buffer, pH 7.5, containing 10% (w/v) sucrose. Specific binding was calculated as the difference between the amount of $^{125}$I-VIP bound in the absence (total binding) and presence (nonsaturable binding) of 1 $\mu$M unlabeled VIP. Nonsaturable $^{125}$I-VIP binding comprised 10 to 15% and 20 to 25% of total binding in rat and human tissues, respectively. Each binding measurement within one experiment was performed in duplicate. The constants $K_i$ for the inhibition of $^{125}$I-VIP binding by unlabeled peptides were calculated from the concentration of unlabeled peptide that produces a 50% inhibition ($IC_{50}$) of the specific $^{125}$I-VIP binding using the following relation:

$$K_i = IC_{50} \frac{K_d}{K_d + L}$$

where $K_d$ is the dissociation constant and $L$ the concentration of $^{125}$I-VIP. Since the concentration of ligand, i.e., 0.05 nM, was low as compared to the $K_d$ of the high affinity VIP binding sites in rat (15) and human (27) intestine, e.g., about 1 nM, $IC_{50}$ is a good estimation of $K_i$. The binding potency relative to that of VIP was then calculated for each analog as

$$\frac{K_i\text{VIP}}{K_i\text{A}} \times 100$$

where $K_i$VIP and $K_i$A are the inhibition constants of unlabeled VIP and analog, respectively. The statistical significance of the difference between $K_i$ values was assessed by the Student's $t$ test.

**Adenylate cyclase assay.** Adenylate cyclase was assayed as follows. In a final volume of 250 $\mu$l the standard incubation medium contained 60 mM HEPES buffer, pH 7.5, 0.4% (w/v) bovine serum albumin, 0.1% (w/v) bacitracin, 0.3 mM ATP, 20 mM creatine phosphate, 1 mg/ml of creatine kinase, 5 mM $MgCl_2$, 0.2 mM 3-isobutyl-1-methylxanthine, and 10 $\mu$M GTP. The incubation was performed for 15 min at 30°. The reaction was started by adding membranes (40–50 $\mu$g of protein/ml). It was stopped and cyclic AMP was determined by radioimmunoassay as described (28). Each measurement within one experiment was performed in duplicate. Each experiment consisted of full concentration-response curves for VIP, GRF, and its analogs. From these curves (see Fig. 2), the concentrations of peptides which induced half the maximal response elicited by VIP ($EC_{50}$) were determined. Potencies relative to that of VIP were calculated as

$$\frac{EC_{50}\ \text{VIP}}{EC_{50}\text{A}} \times 100$$

where $EC_{50}$VIP and $EC_{50}$A represent half-maximally effective concentrations of VIP and analog, respectively.

TABLE 1
**List of GRF analogs**

| Compound | Abbreviation |
|---|---|
| Rat growth hormone-releasing factor | rGRF |
| Human growth hormone-releasing factor-44-$NH_2$ | hGRF |
| (1-Histidine) Growth hormone-releasing factor-40-OH | His$^1$GRF |
| (1-Phenylalanine) Growth hormone-releasing factor-40-OH | Phe$^1$hGRF |
| (1-Tryptophan) Growth hormone-releasing factor-40-OH | Trp$^1$hGRF |
| (1-Alanine) Growth hormone releasing factor-40-OH | Ala$^1$hGRF |
| (1-Acetyl tyrosine) Growth hormone releasing factor-40-OH | Ac-Tyr$^1$hGRF |

## Results

Fig. 1 shows the competitive inhibition of $^{125}$I-VIP binding to human (top) and rat (bottom) intestinal membranes by unlabeled VIP, rGRF, hGRF, and position 1 substituted analogs of hGRF. In human tissue, the dose effects of all peptides were parallel and the following order of potency was observed: VIP > rGRF > His$^1$hGRF > Ac-Tyr$^1$hGRF ≧ Trp$^1$hGRF ≧ hGRF ≧ Phe$^1$hGRF > Ala$^1$hGRF. In rat tissue, the dose effects of all peptides were parallel, also, but the order of potency of the peptides was slightly different from that observed in humans, i.e., VIP > rGRF > His$^1$hGRF ≧ Ac-Tyr$^1$hGRF ≧ Trp$^1$hGRF > hGRF ≧ Phe$^1$hGRF > Ala$^1$hGRF. The intrinsic $K_i$ of each peptide in humans and rats is shown in Table 2. It appears that in both species rGRF has a higher affinity than hGRF for VIP receptors. This is due in part, but not solely, to the presence of an N-terminal histidine in rGRF in the place of tyrosine in hGRF, since the affinity of His$^1$hGRF is higher than that of hGRF but lower than that of rGRF in both species (Table 2).

Fig. 2 shows the stimulation of adenylate cyclase activity in human (top) and rat (bottom) intestinal membranes by the abovementioned peptides. In human and rat tissues, the dose effects of VIP, rGRF, and His$^1$hGRF were parallel and the two latter peptides were VIP agonists of reduced potency (Table 2). In agreement with binding studies, the analysis of adenylate cyclase activation indicates that: 1) rGRF is much more potent than hGRF, and 2) His$^1$hGRF has a potency between those of rGRF and hGRF. In contrast with His$^1$hGRF, other position 1 substituted analogs of hGRF as well as hGRF itself behave as partial VIP agonists of different efficacies. In that respect, the substitution of tyrosine for histidine in position 1 of hGRF has not only increased the potency of the peptide but has also specifically converted a partial agonist, e.g., hGRF, into an agonist, e.g., His$^1$hGRF. The results shown in Fig. 2 also indicate that the efficacies of the partial agonists (measured at 30 $\mu$M peptide) are all higher in human than in rat tissue, e.g.,

45% versus 20% for hGRF, 60% versus 35% for Trp¹hGRF, 55% versus 35% for Phe¹hGRF, 18% versus 10% for Ala¹hGRF, and 20% versus 6% for Ac-Tyr¹hGRF.

The above-described results indicate that the analog Ac-Tyr¹hGRF has a very low intrinsic activity in the rat (about 6% that of VIP), whereas it exhibits a rather high affinity for VIP receptors (see Figs. 1 and 2 and Table 2). This makes Ac-Tyr¹hGRF a good candidate as an antagonist of VIP, at least in the rat. This is tested as shown in Fig. 3. It appears that increasing concentrations of the analog, in the range between 0.3 and 30 $\mu$M, inhibits the VIP-stimulated adenylate cyclase activity. Thirty $\mu$M analog completely abolishes the response induced by 0.01, 0.1, or 1 nM VIP, whereas it partially antagonizes the effect of 10 or 100 nM VIP. The data from five separate experiments are plotted in Fig. 4 according to the method of Arunlakshana and Schild (29), resulting in a straight line. The slope of the regression line (1.05) does not differ significantly from unity, indicating a simple competition mechanism between VIP and Ac-Tyr¹hGRF within the range of concentrations tested. The affinity of the antagonist calculated from the Schild plot ($K_i$) is 350 nM. This inhibition constant compares well with the mean $K_i$ value determined by competition between $^{125}$I-VIP and Ac-Tyr¹hGRF for binding to rat intestinal membranes, i.e., 430 nM (see Table 2).

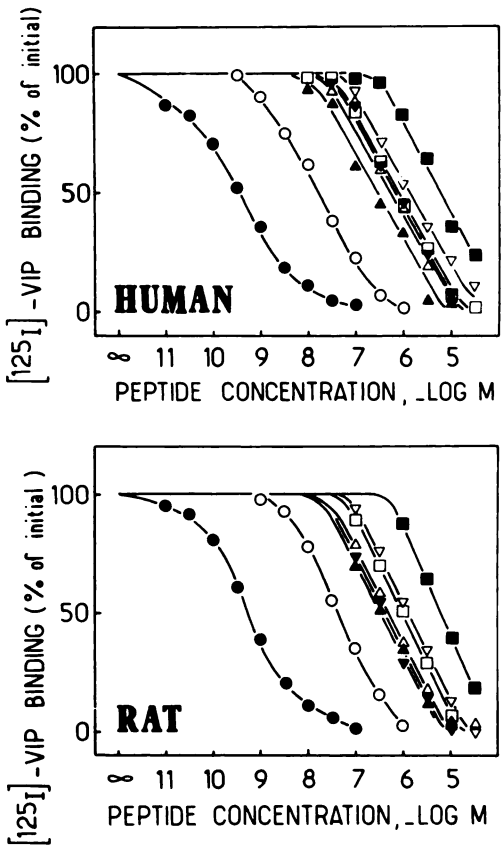


**Fig. 1.** Competitive inhibition of specific $^{125}$I-VIP binding to human and rat intestinal epithelial membranes by VIP, rGRF, hGRF, and postition 1 substituted analogs of hGRF. Conditions were as described in Materials and Methods. Membranes were incubated with the indicated concentrations of VIP (●), rGRF (○), hGRF (□), Ala¹hGRF (■), Ac-Tyr¹hGRF (△), His¹hGRF (▲), Phe¹hGRF (▽), and Trp¹hGRF (▼). Results are expressed as the percentage of radioactivity specifically bound in the absence of added unlabeled peptide. Each point is the mean of four separate experiments. For the sake of clarity, standard errors are not indicated. They were always below 15% of mean values.

## Discussion

The present results clearly demonstrate that rGRF has a 24- and 32-fold higher affinity than hGRF for VIP binding sites in rat and human intestine, respectively. Among the 8- and 9-amino acids that rGRF and hGRF share with VIP (1, 8, 17), the major difference lies in a common N-terminal histidine in rGRF and VIP in the place of a tyrosine in hGRF. This is certainly a structural basis for the increased affinity of rGRF since 1) the presence of N-terminal histidine was shown previously to be very important for peptide interaction with VIP receptors (12–16), and 2) the analog His¹hGRF also has a higher affinity than hGRF for VIP receptors (see Fig. 1 and Table 2). The N-terminal histidine in rGRF is, however, not sufficient to entirely explain its increased affinity since His¹hGRF has an intermediary affinity between those of rGRF and hGRF. The other structural domains involved in the shift of affinity are difficult to determine from the present results. As far as adenylate cyclase activation is concerned, the N-terminal histidine also appears to be very important for generating a full

TABLE 2

**Comparison of the binding and biological activities of VIP, rGRF, hGRF, and position 1 substituted analogs of hGRF in human and rat intestine**

Conditions are as described in *Materials and Methods* and in the legends to Figs. 1 and 2. $K_i$, $EC_{50}$, and relative potency were calculated as described in *Materials and Methods*. Each value is expresssed as mean ± SEM.

| Compound[a] | Binding assay[b] | | | | Adenylate cyclase assay[c] | | | |
|---|---|---|---|---|---|---|---|---|
| | Human | | Rat | | Human | | Rat | |
| | $K_i$ | Potency | $K_i$ | Potency | $EC_{50}$ | Potency | $EC_{50}$ | Potency |
| | nM | | nM | | nM | | nM | |
| VIP | 0.5 ± 01 | 100 | 0.6 ± 0.1 | 100 | 1.2 ± 0.3 | 100 | 1.5 ± 0.2 | 100 |
| rGRF | 26 ± 8 | 1.9 | 46 ± 4 | 1.3 | 38 ± 9 | 3.2 | 100 ± 24 | 1.5 |
| hGRF | 830 ± 150 | 0.06 | 1,100 ± 250 | 0.05 | >30,000[d] | <0.004 | >30,000[d] | <0.005 |
| Ala¹hGRF | 5,200 ± 1,300 | 0.01 | 7,900 ± 960 | 0.008 | >30,000[d] | <0.004 | >30,000[d] | <0.005 |
| Ac-Tyr¹hGRF | 710 ± 120 | 0.06 | 430 ± 30 | 0.14 | >30,000[d] | <0.004 | >30,000[d] | <0.005 |
| His¹hGRF | 320 ± 80 | 0.16 | 460 ± 50 | 0.13 | 1,025 ± 260 | 0.07 | 1,470 ± 460 | 0.10 |
| Phe¹hGRF | 1,240 ± 220 | 0.03 | 1,730 ± 290 | 0.03 | 3,600 ± 540 | 0.03 | >30,000 | <0.005 |
| Trp¹hGRF | 760 ± 140 | 0.07 | 400 ± 40 | 0.15 | 1,640 ± 340 | 0.07 | >30,000 | <0.005 |

[a] Abbreviations are explained in Table 1.
[b] Four separate experiments performed in duplicate.
[c] Five separate experiments performed in duplicate.
[d] Dose-response curve not parallel to standard VIP.

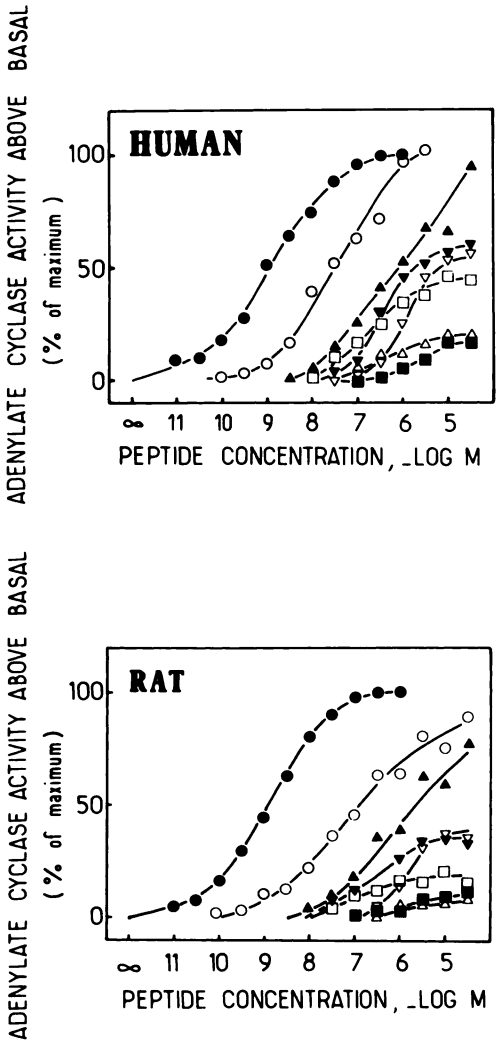


**Fig. 2.** Adenylate cyclase activity in human and rat intestinal epithelial membranes in response to VIP, rGRF, hGRF, and position 1 substituted analogs of hGRF. Conditions are as described in Materials and Methods. Membranes were incubated with the indicated concentrations of VIP (●), rGRF (○), hGRF (□), Ala$^1$hGRF (■), Ac-Tyr$^1$hGRF (△), His$^1$hGRF (▲), Phe$^1$hGRF (▽), and Trp$^1$hGRF (▼). Results are expressed as the percentage of maximum stimulation ($10^{-6}$ M VIP) of cyclic production above basal levels. Each point is the mean of five separate experiments. For the sake of clarity, standard errors are not indicated. They were always below 16% of mean values.

response. Indeed, rGRF and His$^1$hGRF are VIP agonists, whereas hGRF and other position 1 substituted analogs of hGRF behave as partial agonists. These results are in good agreement with previous observations indicating that replacing the N-terminal histidine of secretin (15, 16) or deleting the N-terminal histidine of VIP (14) results in a drastic loss of affinity for rat VIP receptors and generate partial VIP agonists.

We have previously reported that hGRF does not generate physiological response through its interaction with VIP receptors in humans (12). The present results suggest that the same holds true for rGRF in the rat, even though it is much more potent than hGRF is in man. Indeed, rGRF stimulates adenylate cyclase activity through rat VIP receptors at concentrations above 1 nM while its specific effects mediated by GRF receptors, such as the stimulation of growth hormone release *in vitro* (30), are observed at concentrations between 0.01 and 1 nM. A definitive answer to this question has to wait, however, for the determination of local GRF concentrations at the vicinity of intestinal VIP receptors since GRF-like immunoreactivity has been detected in the gastrointestinal tract (31, 32). In contrast with the present results, rGRF was reported to have pronounced VIP-like effects in the guinea pig exocrine pancreas where it is one-half as potent as VIP in stimulating amylase release, an effect apparently mediated by VIP receptors (33). This further emphasizes the important species specificity in the interaction

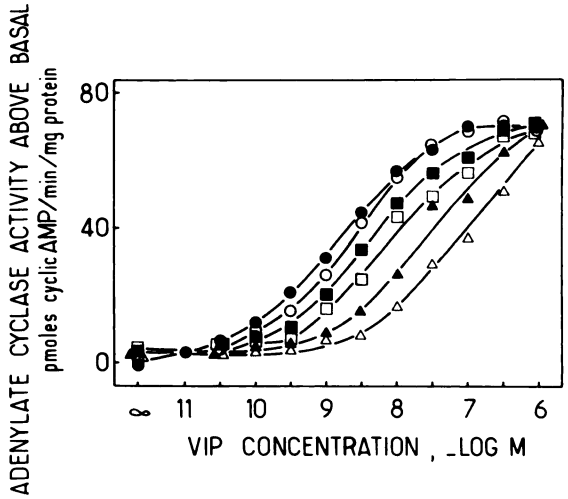


**Fig. 3.** Inhibition by Ac-Tyr$^1$hGRF of adenylate cyclase activity stimulated by various concentrations of VIP in rat intestinal epithelial membranes. Conditions are as described in Materials and Methods. The indicated concentrations of VIP were incubated together with the following concentrations of Ac-Tyr$^1$hGRF: 0 (●), 0.3 μM (○); 1 μM (■), 3 μM (□); 10 μM (▲); 30 μM (△). Each point is the mean of five separate experiments. For the sake of clarity standard errors are not indicated. They were always below 15% of mean values.

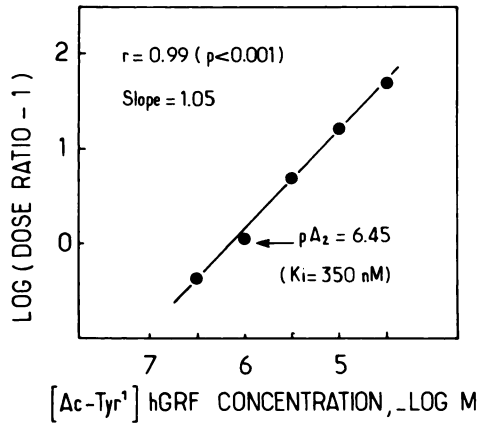


**Fig. 4.** $pA_2$ value for Ac-Tyr$^1$hGRF antagonism of VIP-stimulated adenylate cyclase activity in rat intestinal epithelial membranes. Conditions are as described in Materials and Methods. Adenylate cyclase activity was obtained with 11 separate concentrations of VIP in the absence and presence of Ac-Tyr$^1$hGRF at the concentrations indicated. Antagonism was analyzed by a Schild plot (29) in which antagonism was expressed by the dose ratios of VIP needed to produce half-maximal responses in the absence and presence of different concentrations of Ac-Tyr$^1$hGRF: log (dose ratio − 1) = $n$ log (antagonist) − log $K_i$. The intercept with the abscissa (dose ratio = 2) is the $pA_2$ value (− log $K_i$), i.e., the negative log of the receptor-antagonist apparent dissociation constant. Dose ratios were estimated graphically in five separate experiments as shown in Fig. 3. The regression line was calculated by the method of the least squares.

of VIP-related peptides with VIP receptors. In that respect only studies performed in homologous systems, such as the present one, are relevant to draw a conclusion concerning the physiological or pharmacological implications of peptide-receptor interactions.

The tendency of hGRF to behave as a partial VIP agonist/antagonist in the rat was previously noted (12) and is confirmed here. It is enhanced in some position 1 substituted analogs of hGRF such as in Ala[1]hGRF and especially in Ac-Tyr[1]hGRF. Similar results are obtained with human VIP receptors, but these hGRF analogs as well as hGRF itself have a higher efficacy than in the rat (see Fig. 2). Ac-Tyr[1]hGRF exhibits a negligible intrinsic activity in stimulating the rat intestinal adenylate cyclase activity, e.g., about 6% of that with VIP. This result suggests that a free N-terminal amino group is essential for generating the biological response. Since acetylation of this primary amine rather increases the affinity of hGRF for rat VIP receptors ($K_i$ = 430 nM versus 1100 nM), Ac-Tyr[1]hGRF becomes the first VIP antagonist ever described. The analog does antagonize the stimulation of adenylate cyclase by VIP through a competitive mechanism as indicated by the Schild plot described in Fig. 4. Ac-Tyr[1]hGRF completely antagonizes the action of VIP concentrations up to 1 nM that are in the range of the estimated local concentrations of peptide at the vicinity of peripheral VIP nerve endings (34) and are higher than the circulating levels of VIP in the systemic arterial blood, e.g., 0.01 nM, and even in the hypophyseal portal blood, e.g., 0.3 nM (35). In that respect, Ac-Tyr[1]hGRF may be a useful tool for understanding the physiological implications of VIP in the rat, but with two limitations: 1) it has a residual intrinsic activity mediated by VIP receptors, and 2) Ac-Tyr[1]hGRF is a GRF agonist of low potency, e.g., 10% of that for rGRF in the rat (18). In any case, the present results represent the first description of a VIP antagonist and pave the way of the synthesis of a potent VIP antagonist by modification of VIP itself.

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