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Characterization of VIP receptor-effector system antagonists in rat and mouse peritoneal macrophages

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Abstract

In the present study we show that the synthetic peptides [4-Cl-D-Phe^6,Leu^{17}]VIP and the growth hormone releasing factor (GRF) analog [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2 inhibit in a competitive manner the specific [^{125}I]VIP binding to both rat and mouse peritoneal macrophages. In rat peritoneal macrophages, the order of potency of the different peptides, as expressed by the IC_{50} values was: VIP ($IC_{50} = 1.90 \pm 0.16$ nM) > [4-Cl-D-Phe^6,Leu^{17}]VIP ($IC_{50} = 125.8 \pm 13.2$ nM) > [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2 ($IC_{50} = 354.8 \pm 21.2$ nM). In mouse peritoneal macrophages a similar pattern of potency was observed: VIP ($IC_{50} = 1.58 \pm 0.12$ nM) > [4-Cl-D-Phe^6,Leu^{17}]VIP ($IC_{50} = 110.8 \pm 10.7$ nM) > [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2 ($IC_{50} = 251 \pm 19.2$ nM). The behavior as VIP receptor antagonists of both [4-Cl-D-Phe^6,Leu^{17}]VIP and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2 in rat and mouse peritoneal macrophages was confirmed by: (a) the shift to the right of VIP dose-stimulated cyclic AMP production curves in the presence of the two antagonists; (b) the agreement between the order of efficacy of the two peptides in competition experiments with the corresponding inhibition of cyclic AMP production; (c) the inefficiency of the two antagonists on the stimulation of cyclic AMP production by the β-adrenoceptor agonist isoproterenol, which indicates the specificity of the interaction; (d) the synergic effect of VIP on isoproterenol-stimulated cyclic AMP production was completely abolished by [4-Cl-D-Phe^6,Leu^{17}]VIP or [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2, suggesting that both antagonists acted via specific VIP receptors. Moreover, propranolol, a β-adrenoceptor antagonist, did not affect the VIP-stimulated cyclic AMP production and the antagonist role of [4-Cl-D-Phe^6,Leu^{17}]VIP or [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2; (e) in cross-linking experiments, the intensity of the labeling of the [^{125}I]VIP/receptor complexes was significantly lower with the antagonists than in the control experimental situation in both mouse and rat peritoneal macrophage membranes.

Keywords: VIP (vasoactive intestinal peptide); Peritoneal macrophage; Adenylyl cyclase; VIP receptor antagonist; VIP receptor; Propranolol; Isoproterenol; Neuroimmunobiology

1. Introduction

Vasoactive intestinal peptide (VIP) is a 28-amino-acid peptide of the glucagon-secretin family of peptides that is widely distributed in the organism (Said and Mutt, 1970). VIP has many pharmacological effects that include local regulation of blood flow, smooth muscle relaxation, exocrine gland secretion and neuroimmunomodulation of several immune functions (Said, 1991; Ottaway, 1991). With respect to immune system, numerous studies have implicated VIP as an immunoregulatory peptide in both humans and experimental animals (Tseng and O'Dorisio, 1989; Ottaway, 1991). In this context, specific high affinity receptors for VIP have been identified on human peripheral blood lymphocytes (Guerrero et al., 1981; Ottaway et al., 1983, 1990; Calvo et al., 1986a), human monocytes (Wiik et al., 1985), rat lymphoid cells (Calvo et al., 1986b), and rat and mouse peritoneal macrophages (Segura et al., 1991; Calvo et al., 1994a,b). On the other hand, VIP has been shown to activate adenylyl cyclase in human lymphocytes membranes (O'Dorisio et al., 1981), and to stimulate cyclic AMP production in peripheral blood lymphocytes (Guerrero et al., 1981; Calvo et al., 1986a) and in rat and mouse peritoneal macrophages (Segura et al., 1992a; Pozo et al., 1996). Moreover, recently we have shown that VIP inhibits substrate adherence capacity of rat peritoneal macrophages by a cAMP mediated mechanism (Segura et al., 1993).

The ability to assess the physiological importance of VIP in different immunological processes is limited by the

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lack of specific potent antagonists. A number of different classes of VIP antagonists have been described including D-amino-substituted analogues of VIP, such as [4-Cl-D-Phe^6,Leu^{17}]VIP (Pandol et al., 1986); amino-substituted analogues of the structurally related peptide growth hormone releasing factor (GRF), such as [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2 (Waelbroeck et al., 1985; Laburthe et al., 1986); COOH terminal fragments of VIP, such as VIP(10–28) (Bissonnette et al., 1984); chimeric analogues, such as neurotensin(6–11)-VIP(7–28) (Gozes et al., 1991) or more recently VIP(6–28)-PACAP(28–38) (Fishbein et al., 1994), and recently a human VIP analog has been designed in which the residues 12–19 were replaced by a spacer of the same length, $(\gamma$-aminobutyryl$)_2$ (Leroux et al., 1994). Several studies have been realized with these antagonists. Thus, [4-Cl-D-Phe^6,Leu^{17}]VIP has been reported to be a specific antagonist of VIP in rat pancreas (Pandol et al., 1986), intestine (Grider and Rivier, 1990; Espat et al., 1995), seminal vesicle (Rodríguez-Pena et al., 1991), and in heart dog (Hill et al., 1995). Growth hormone releasing factor (GRF) analog [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2 is an effective and specific VIP receptor antagonist in rat pancreas (Waelbroeck et al., 1985), intestine (Laburthe et al., 1986), seminal vesicle (Rodríguez-Pena et al., 1991), spinal cord (Xu and Wiesenfeld-Hallin, 1991), and in cat colon (Blank et al., 1990). Finally, neurotensin(6–11)-VIP(7–28) has been shown to be a potent VIP receptor antagonist in rat central nervous system (Gozes et al., 1991; Lilling et al., 1995).

With respect to effects of these VIP receptor antagonists on the immune system, very little information is available. Thus, only it is known that [4-Cl-D-Phe^6,Leu^{17}]VIP is able to reverse the inhibitory effect of VIP on human natural killer activity (Sirianni et al., 1992), to compete the effects of VIP on cyclic AMP production and concanavalin A stimulated proliferation of mouse lymphocytes (Ottaway, 1992), and to inhibit substrate adherence capacity of rat peritoneal macrophages (Segura et al., 1993).

An antagonist is a good tool to define the specific responses to VIP in cells and tissues that possesses specific VIP receptors. In this paper we report the effects of [4-Cl-D-Phe^6,Leu^{17}]VIP and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2, two important VIP receptor antagonists in rat and mouse peritoneal macrophages.

2. Materials and methods

2.1. Chemicals

Synthetic rat VIP, [4-Cl-D-Phe^6,Leu^{17}]VIP, and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2 was purchased from Peninsula Laboratories Europe (Merseyside, UK). Leupeptine, phenylmethylsulfonylfluoride, and N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), were from Boehringer-Mannheim (Mannheim, Germany). Bacitracin, bovine serum albumin (fraction V), 3-isobutyl-1-methyl-xanthine (IBMX), ethylenediaminetetraacetic acid (EDTA), *N*-[2-hydroxyethyl]piperazine-*N*′-[2-hydroxypropanesulfonic acid] (Hepes), (−)-isoproterenol, and D,L-propranolol were purchased from Sigma (St. Louis, MO, USA). Electrophoretic chemicals and molecular mass standards were from Bio-Rad Laboratories (Richmond, CA, USA). Dithiobis (succinimidyl propionate) (DTSP) was from Pierce (Rockford, IL, USA). Carrier-free $Na^{125}I$ (IMS 30, 100 mCi/ml) and cyclic AMP assay kits were obtained from Radiochemical Center (Amersham, UK). Synthetic rat [^{125}I]VIP was prepared as previously described (Calvo et al., 1986a) with a specific activity of about 800 Ci/mmol. All other chemicals were reagent grade.

2.2. Collection of rat and mouse peritoneal macrophages

Rat and mouse peritoneal macrophages were elicited by the method described in detail previously (Segura et al., 1991). Utmost precautions were taken such that the animals remained free from infection by environmental pathogens. Briefly, animals allowed free access to food and water, were injected intraperitoneally 4 days before harvest with 6% sodium caseinate. Animals were killed by decapitation and, immediately, peritoneal cavity was washed with cold 0.15 M NaCl. Cells were pelleted by centrifugation, resuspended in 0.15 M NaCl and immediately used for experiments. Viability, as determined by Trypan blue exclusion, was always greater than 95%.

2.3. Rat and mouse peritoneal macrophage membrane preparation

Macrophage membranes were obtained as described previously (Segura et al., 1992b). Briefly, macrophages were resuspended in 5 mM Hepes (pH 7.5) containing 0.1 mg/ml bacitracin, 0.01 mg/ml leupeptine, 0.01 mg/ml TLCK, 0.05 mg/ml phenylmethylsulfonylfluoride and 1 mM EDTA. After 15 min incubation at 4°C, cells were disrupted by sonication for two 10-s bursts at maximal power and tune meter separated by 10-s intervals. The homogenate was centrifugated at $600 \times g$ for 10 min at 4°C. The $600 \times g$ supernatant was centrifugated at $30\,000 \times g$ for 30 min at 4°C. The $30\,000 \times g$ pellet was resuspended in 20 mM Hepes containing 0.05 mg/ml phenylmethylsulfonylfluoride and was immediately frozen at −80°C until used. Proteins were measured by the method of Bradford (1976) using bovine serum albumin as standard.

2.4. Binding studies

In standard conditions, rat and mouse peritoneal macrophages (1.5×10^6 cells/ml) were incubated at 15°C in 0.5 ml of 35 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl, 1.4% (w/v) bovine serum albumin, 1

mg/ml bacitracin and 45 pM [^{125}I]VIP either alone or together with increasing concentrations of unlabelled VIP (0.01–100 nM), [4-Cl-D-Phe^6,Leu^{17}]VIP (0.1–1000 nM) or [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2 (0.1–1000 nM). After 90 min incubation, cell-bound peptide was separated by centrifugation, as described previously (Calvo et al., 1986a), and the radioactivity associated with the cells was measured in a Pharmacia gamma counter. Specific binding was calculated from total binding by subtracting nonspecific binding, as determined by binding of tracer in the presence of unlabelled VIP at 1 μM. Nonspecific binding was about 3–4% of the total radioactivity added.

The IC_{50} (the competitor concentration at which the specific binding of the tracer is reduced by 50%) values were used to calculate the equilibrium dissociation constant (K_i) for each unlabeled peptide, following the equation: $K_i = IC_{50}[K_d/(K_d + F)]$, where K_d is the dissociation constant of the high affinity VIP binding sites in rat and mouse peritoneal macrophages and F is the concentration of [^{125}I]VIP.

2.5. Cyclic AMP production

Cyclic AMP was determined as previously described (Segura et al., 1992a). In a standard assay, rat and mouse peritoneal macrophages (10^6 cells/ml) were incubated at 15°C in 0.5 ml of 35 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl, 1.4% (w/v) bovine serum albumin, 1 mg/ml bacitracin, 0.2 mM 3-isobutyl-1-methylxanthine (IBMX) in the absence or presence of test substances (VIP, VIP receptor antagonist, isoproterenol or propranolol). After 45 min incubation, the reaction was stopped by the addition of 2.5 ml methanol. The precipitate was removed by centrifugation, aliquots of the supernatant were evaporated and cyclic AMP was measured by a kit cyclic AMP assay system.

ED_{50} represents the concentration of VIP that induced a half-maximal response upon the cyclic AMP production. The corresponding K_i value was determined from the equation for antagonist inhibition of agonist stimulation: K_i = [antagonist]/([EC_{50} (A)/EC_{50} (C)] − 1) where [antagonist] is the concentration of antagonist and EC_{50} (A) and EC_{50} (C) are the concentrations of VIP which induced half-maximal response in the presence and absence of antagonist, respectively (Furchgott, 1967).

2.6. Cross-linking of [^{125}I]VIP to rat and mouse macrophage membranes

Rat and mouse macrophage membranes (100 μg/ml) were incubated for 60 min at 15°C in 5 ml of 20 mM Hepes buffer (pH 7.5) containing 2% (w/v) bovine serum albumin and 0.1% (w/v) bacitracin, with [^{125}I]VIP (0.3 nM) and [4-Cl-D-Phe^6,Leu^{17}]VIP (100 nM) or [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2 (100 nM). To determine nonspecific binding, parallel samples were incubated as above, but in the presence of an excess (1 μM) of unlabelled VIP. After incubation, the reaction was stopped by adding 25 ml of ice-cold 20 mM Hepes buffer (pH 7.5). Membrane-bound [^{125}I]VIP was separated at 4°C by centrifugation at 30 000 × g for 15 min. The supernatant was withdrawn and the pellet was then resuspended in 1 ml of the same buffer, but including 1 mM DTSP. The reaction was carried out for 15 min at 4°C and was stopped by adding 20 μl of ice-cold 20 mM Hepes buffer, pH 7.5, containing 60 mM glycine as a reagent quench (Calvo et al., 1994a). The mixture was centrifuged at 4°C for 15 min at 30 000 × g, and the resulting pellet was suspended by five successive passages through a 25-gauge needle in 60 mM Tris-HCl buffer (pH 8.8) containing 10% (v/v) glycerol, 0.001% (w/v) bromophenol blue and 3% (w/v) sodium dodecyl sulfate (SDS). After heating for 30 min at 60°C, the suspension was centrifuged for 15 min at 48 000 × g and the supernatant was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.7. SDS-PAGE and autoradiography

SDS-PAGE was carried out according to the method of Laemmli (1970) in a 10% polyacrylamide slab gel (1.5 mm thickness) with a 5% polyacrylamide stacking gel. Calibration proteins were: phosphorylase *b* (107 kDa), bovine serum albumin (76 kDa), ovalbumin (52 kDa), carbonic anhydrase (36 kDa), soybean trypsin inhibitor (27.8 kDa) and lysozyme (19 kDa). The gels were dried and exposed for about 8–12 days at −80°C to a DuPont Cronex-4 film with an intensifying screen (DuPont Cronex Lightning Plus).

2.8. Calculations and statistics

Binding data were analyzed by the method of Scatchard (1949) using the nonlinear curve-fitting program LIGAND (Munson and Rodbard, 1980). The results are presented as means ± S.E.M. Statistical significance was analyzed by one way analysis of variance (ANOVA).

3. Results

3.1. Effect of VIP, [4-Cl-D-Phe^6,Leu^{17}]VIP and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2 on [^{125}I]VIP binding and cyclic AMP production in rat and mouse peritoneal macrophages

In competition studies, the specific binding of [^{125}I]VIP to rat and mouse peritoneal macrophages was inhibited by increasing concentration on unlabelled VIP, [4-Cl-D-Phe^6,Leu^{17}]VIP and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2 (Fig. 1). In rat peritoneal macrophages (Fig. 1, left) the order of potency of the different peptides, as expressed by the concentration giving half-maximal inhibition of tracer

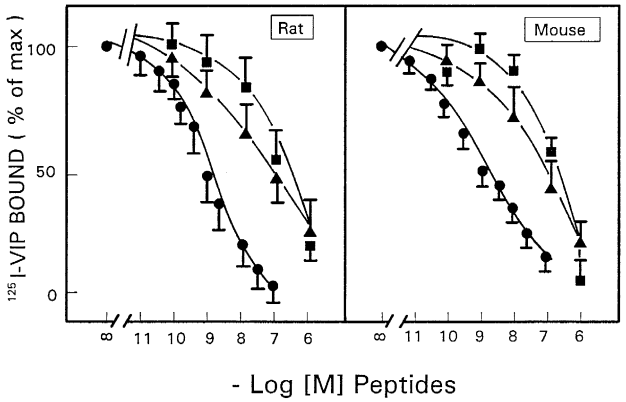

Fig. 1. Competitive inhibition of [^{125}I]VIP binding to rat (left) and mouse (right) peritoneal macrophages. Cells (1.5×10^6 cells/ml) were incubated with 45 pM [^{125}I]VIP at 15°C for 90 min in the presence of increasing concentrations of unlabelled VIP (●), [4-Cl-D-Phe^6,Leu^{17}]VIP (▲) and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH$_2$ (■). Specific binding is expressed as the percentage of maximun binding measure in the presence of tracer alone. Each point is the mean ± standard error of five separate experiments performed in triplicate.

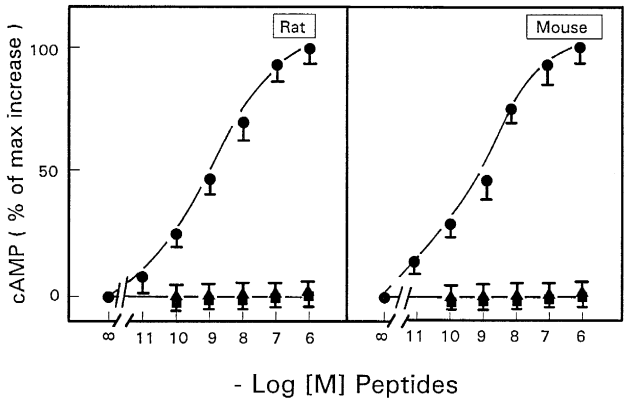

Fig. 2. Effect of increasing concentrations of VIP (●), [4-Cl-D-Phe^6,Leu^{17}]VIP (▲) and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH$_2$ (■) on cyclic AMP production in rat (left) and mouse (right) peritoneal macrophages. Cells (10^6/ml) were incubated for 45 min at 15°C with different concentration of indicated peptide. Results are expressed as percentages of maximum cyclic AMP increment above basal. Each point is the mean ± standard error of five separate experiments performed in triplicate.

binding, was as follows: VIP ($IC_{50} = 1.90 \pm 0.16$ nM) > [4-Cl-D-Phe^6,Leu^{17}]VIP ($IC_{50} = 125.8 \pm 13.2$ nM) > [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH$_2$ ($IC_{50} = 354.8 \pm 21.2$ nM). In mouse peritoneal macrophages (Fig. 1, right), we observed a similar pattern of potency: VIP ($IC_{50} = 1.58 \pm 0.12$ nM) > [4-Cl-D-Phe^6,Leu^{17}]VIP ($IC_{50} = 110.8 \pm 10.7$ nM) > [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH$_2$ ($IC_{50} = 251 \pm 19.2$ nM). Moreover, in mouse peritoneal macrophages, the effect of [4-Cl-D-Phe^6,Leu^{17}]VIP and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH$_2$ on specific [^{125}I]VIP binding was statistically different ($P < 0.05$, ANOVA test) and greater than in the rat, as shown in Table 1. VIP-stimulated cyclic AMP production in both rat and mouse peritoneal macrophages is shown in Fig. 2. Furthermore, the two peptides examined were ineffective in modifying the basal cyclic AMP levels when tested at concentration as high as 1 μM. These results suggest that the two peptides studied might have VIP receptor antagonist properties.

3.2. Effect of [4-Cl-D-Phe^6,Leu^{17}]VIP and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH$_2$ on VIP-stimulated cyclic AMP production in rat and mouse peritoneal macrophages

The antagonist properties of [4-Cl-D-Phe^6,Leu^{17}]VIP and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH$_2$ were studied by the magnitude of the shift of the VIP-stimulated cyclic AMP production in the presence of 10 μM of one of the tested peptides. [4-Cl-D-Phe^6,Leu^{17}]VIP and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH$_2$ modified the ED_{50} of VIP and impaired its maximal effect in a dose-dependent manner in both rat (Fig. 3, left) and mouse (Fig. 3, right) peritoneal macrophages. In the presence of the two synthetic peptides, was observed a marked shift to the right in the dose-effect curves of VIP-stimulated cyclic AMP production in the following manner: VIP (ED_{50} rat = 1.25 ± 0.2 nM, ED_{50} mouse = 0.9 ± 0.15 nM) > [4-Cl-D-

Table 1
Ability of [4-Cl-D-Phe^6,Leu^{17}]VIP and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH$_2$ to inhibit VIP-stimulated cAMP accumulation and [^{125}I]VIP binding in both rat and mouse peritoneal macrophages

	[^{125}I]VIP binding		cAMP accumulation	
	IC_{50} (nM)	K_i (nM)	ED_{50} (nM)	K_i (nM)
Rat				
VIP	1.900 ± 0.16		1.25 ± 0.2	
VIP-A-1	125.8 ± 13.2	120.6	63.0 ± 5.3	202.4
VIP-A-2	354.8 ± 21.2	340.6	31.6 ± 4.5	411.8
Mouse				
VIP	1.580 ± 0.12		0.90 ± 0.15	
VIP-A-1	110.8 ± 10.7	106.2	44.0 ± 3.7	209
VIP-A-2	251.0 ± 19.2	240.7	17.8 ± 1.2	532.5

Data were calculated according to the equations in Section 2 from the results of five experiments performed in triplicate (see Fig. 3Fig. 4). Values are the means ± S.E. VIP-A-1 and VIP-A-2 are [4-Cl-D-Phe^6,Leu^{17}]VIP and [Ac-Tyr^1,D-Phe^2]GRF-1(1–29)-NH$_2$, respectively.

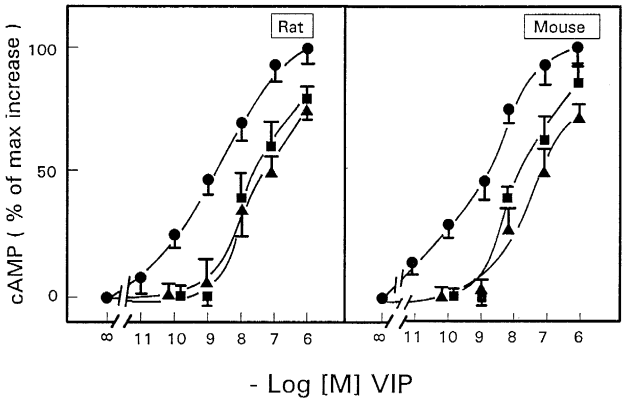

Fig. 3. Effect of 10 μM [4-Cl-D-Phe^6,Leu^{17}]VIP (▲) and 10 μM [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2 (■) on the dose-response curve of VIP-stimulated cAMP accumulation (●) in rat (left) and mouse (right) peritoneal macrophages. Cells (10^6/ml) were incubated for 45 min at 15°C with different concentration of indicated peptides. Results are expressed as percentages of maximum cyclic AMP increment above basal. Each point is the mean ± standard error of five separate experiments performed in triplicate.

Phe^6,Leu^{17}]VIP (ED_{50} rat = 63.0 ± 5.3 nM, ED_{50} mouse = 44.0 ± 3.7 nM) > [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2 (ED_{50} rat = 31.6 ± 4.5 nM, ED_{50} mouse = 17.8 ± 1.2 nM). As shown in Table 1, statistically differences ($P < 0.05$, ANOVA test) were founded between rat and mouse VIP receptor-effector responses. The ability of the two peptides to attenuate VIP-stimulated cyclic AMP production was further investigated by incubating increasing concentration of [4-Cl-D-Phe^6,Leu^{17}]VIP and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2 in the presence of 1 nM of VIP. As shown in Fig. 4, the concentrations of [4-Cl-D-Phe^6,Leu^{17}]VIP and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2 that reduced the maximal value by 50% were 40 nM and 79.4 nM in rat (Fig. 4, right) and 30 nM and 95 nM in mouse (Fig. 4, left), respectively.

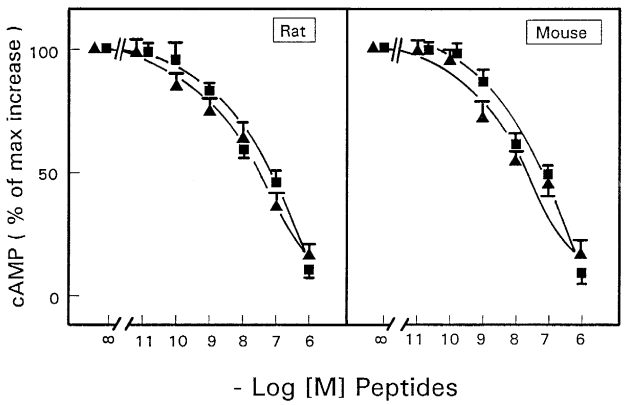

Fig. 4. Effect of increasing concentrations of [4-Cl-D-Phe^6,Leu^{17}]VIP (▲) and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2 (■) on cAMP accumulation in rat (left) and mouse (right) peritoneal macrophages stimulated by 1 nM VIP. Values are the mean ± standard error of five separate experiments performed in triplicate and are expressed as percentages of the activity observed in the absence of any antagonist.

Table 2
Effects of β-adrenoceptor agonist and adrenoceptor blocker on the inhibitory effect of [4-Cl-D-Phe^6,Leu^{17}]VIP and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2 on the VIP receptor-effector system in rat and mouse peritoneal macrophages

	cAMP accumulation (pmol/10^6 cells)	
	Rat	Mouse
Control	2.3 ± 0.7 [a]	2.1 ± 0.9 [b]
Iso [k]	21 ± 3 [c]	22.12 ± 2 [d]
Iso + VIP-A-1 [l]	20.4 ± 2.3	21.45 ± 0.8
Iso + VIP-A-2 [l]	22.1 ± 1.6	21.8 ± 2
Iso + VIP [m]	33.02 ± 5.2 [e]	31.3 ± 2.9 [f]
Iso + VIP + VIP-A-1 [n]	23.72 ± 2	25.11 ± 2.5
Iso + VIP + VIP-A-2 [n]	25.03 ± 1.9	26 ± 1.3
Prop [o]	2.63 ± 0.1 [g]	3.15 ± 0.5 [h]
Prop + VIP [p]	7.36 ± 0.2 [i]	8.42 ± 1.1 [j]
Prop + VIP + VIP-A-1 [q]	2.89 ± 1.02	3.42 ± 0.8
Prop + VIP + VIP-A-2 [q]	3.28 ± 1.2	3.15 ± 0.1

[k] $P < 0.05$ vs. [a] and [b], respectively; [l] $P > 0.05$ vs. [c] and [d], respectively; [m] $P < 0.05$ vs. [c] and [d], respectively; [n] $P < 0.05$ vs. [e] and [f], respectively; [o] $P > 0.05$ vs. [a] and [b], respectively; [p] $P < 0.05$ vs. [g] and [h], respectively; [q] $P < 0.05$ vs. [i] and [j], respectively. Values are means ± S.E.M. Statistical significance was analyzed by one way analysis of variance (ANOVA). Iso, 10 μM isoproterenol; VIP-A-1 and VIP-A-2, 10 μM of [4-Cl-D-Phe^6,Leu^{17}]VIP and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2, respectively; VIP, 0.1 μM vasoactive intestinal peptide; Prop, 10 μM propanolol.

The dissociation constant (K_i) for the antagonist derived from radioligand binding experiments and cyclic AMP production (Table 1) was obtained by the equations presented in Section 2.

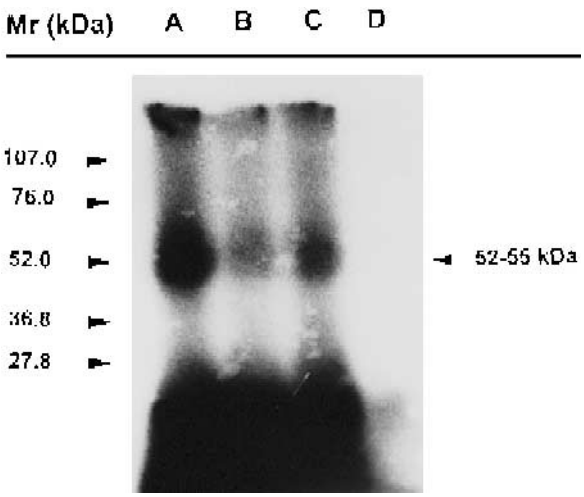

Fig. 5. Effect of [4-Cl-D-Phe^6,Leu^{17}]VIP and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2 on cross-linking of [^{125}I]VIP to rat peritoneal macrophages. Rat peritoneal macrophage membranes were incubated with [^{125}I]VIP as specified in Section 2. After binding reactions and treatment with 1 mM DTSP, membranes were solubilized under non-reducing conditions and subjected to 10% SDS-PAGE. Lanes A and D correspond to [^{125}I]VIP incubations in the absence or presence of 1 μM VIP, respectively. Lanes B and C correspond to [^{125}I]VIP incubations in the presence of 100 nM [4-Cl-D-Phe^6,Leu^{17}]VIP or 100 nM [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2, respectively. The autoradiogram of a representative dried gel is shown.

3.3. Effects of β-adrenoceptor agonist and adrenoceptor antagonist on the inhibitory effect of [4-Cl-D-Phe^6,Leu^{17}]VIP and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH$_2$ on the VIP receptor-effector system in both rat and mouse peritoneal macrophages

The specificity of the inhibitory effect of the two antagonists upon the VIP receptor-effector system was studied. The following experiments compared the effect of isoproterenol, a β-adrenoceptor agonist, on cyclic AMP production in the presence or absence of 10 μM [4-Cl-D-Phe^6,Leu^{17}]VIP or 10 μM [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH$_2$ in rat and mouse peritoneal macrophages. As shown in Table 2, these peptides did not inhibit the β-adrenoceptor stimulation by isoproterenol. Moreover, the synergic effect of 0.1 μM VIP on isoproterenol-stimulated cyclic AMP production was completely abolished by 10 μM of [4-Cl-D-Phe^6,Leu^{17}]VIP or 10 μM [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH$_2$ in both rat and mouse peritoneal macrophages (Table 2). These results demonstrated that [4-Cl-D-Phe^6,Leu^{17}]VIP and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH$_2$ acted via specific VIP receptors.

As shown in Table 2, propranolol, a β-adrenoceptor antagonist, did not affect the VIP-stimulated cyclic AMP production and the antagonist role of [4-Cl-D-Phe^6,Leu^{17}]VIP or [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH$_2$.

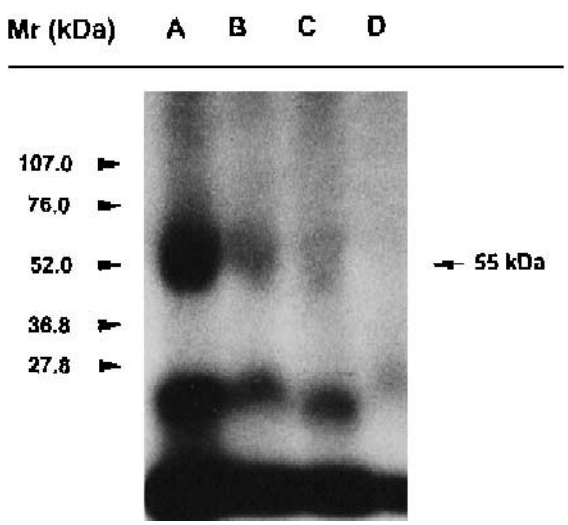

Fig. 6. Effect of [4-Cl-D-Phe^6,Leu^{17}]VIP and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH$_2$ on the labeling of the 55-kDa band. Mouse peritoneal macrophage membranes were incubated with [^{125}I]VIP as specified in Section 2. After binding reactions and treatment with 1 mM DTSP, membranes were solubilized under non-reducing conditions and subjected to 10% SDS-PAGE. Lanes A and D correspond to [^{125}I]VIP incubations in the absence or presence of 1 μM VIP, respectively. Lanes B and C correspond to [^{125}I]VIP incubations in the presence of 100 nM [4-Cl-D-Phe^6,Leu^{17}]VIP or 100 nM [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH$_2$, respectively. The autoradiogram of a representative dried gel is shown.

3.4. Effect of [4-Cl-D-Phe^6,Leu^{17}]VIP and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH$_2$ on cross-linking of [^{125}I]VIP to rat and mouse peritoneal macrophage membranes

To further analyze the effect of both antagonists at molecular level, [^{125}I]VIP was covalently cross-linked to the receptor in the absence and presence of 100 nM [4-Cl-D-Phe^6,Leu^{17}]VIP or 100 nM of [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH$_2$. After the cross-linked materials were subjected to SDS-PAGE, autoradiography revealed a major band, corresponding to the migration of a protein of 52–55 kDa in rat peritoneal macrophage membranes (Fig. 5) and 55 kDa (Fig. 6) in mouse peritoneal macrophage membranes as previously described (Calvo et al., 1994a,b). The intensity of the labeling of the [^{125}I]VIP/receptor complexes was significantly lower with the antagonists than in the control experimental situation in both mouse and rat peritoneal macrophage membranes. The specificity of the 55 kDa and 52–55 kDa [^{125}I]VIP-binding components was determined in the presence of 1 μM unlabelled VIP.

4. Discussion

The present paper show that [4-Cl-D-Phe^6,Leu^{17}]VIP and the GRF-substituted analog [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH$_2$ behave as competitive VIP receptor antagonists in rat and mouse peritoneal macrophages. It is known that the N-terminal histidine residue of VIP plays an important role in the interaction of the peptide with its receptor (Couvineau et al., 1985; Laburthe et al., 1986; Nau et al., 1987) and in the subsequent activation of adenylate cyclase (Robberecht et al., 1984; Laburthe et al., 1986). In fact, the potency of VIP decreased markedly when this histidine residue is modified (Robberecht et al., 1984; Laburthe et al., 1986) or removed (Couvineau et al., 1985; Nau et al., 1987). It has been shown that N-terminal-modified analogs of GRF can act as antagonists of the VIP receptor-effector system, e.g., [Ac-Tyr^1]hGRF (Laburthe et al., 1986; Cox and Cuthbert, 1989) and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH$_2$ (Waelbroeck et al., 1985; Cox and Cuthbert, 1989).

The present results show that [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH$_2$ and [4-Cl-D-Phe^6,Leu^{17}]VIP bind specifically to VIP receptors present in rat and mouse peritoneal macrophages with low affinity as expressed by their order of efficacy: VIP ($IC_{50} = 1.90 \pm 0.16$ nM) > [4-Cl-D-Phe^6,Leu^{17}]VIP ($IC_{50} = 125.8 \pm 13.2$ nM) > [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH$_2$ ($IC_{50} = 354.8 \pm 21.2$ nM).

Both [4-Cl-D-Phe^6,Leu^{17}]VIP and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH$_2$ VIP receptor antagonists in rat and mouse peritoneal macrophage membranes bind to VIP receptors (Fig. 1) but do not stimulate cyclic AMP produc-

tion (Fig. 2). It is possible that the GRF-derived antagonist could inhibit the cyclic AMP production through GRF-preferring receptors, but just at present, GRF receptors have not been described in macrophages. However, it is interesting to indicate that human monocytes specifically bound radiolabeled growth hormone (GH) and contained mRNA for the GH receptor (Warwick-Davies et al., 1995). Further investigations are necessary to demonstrate the existence of specific GRF receptors on macrophages.

Our results indicate that the actions of both [4-Cl-D-Phe^6,Leu^{17}]VIP and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2 are specifically mediated through VIP receptors present in rat and mouse peritoneal macrophages by: (a) the shift to the right of VIP dose-stimulated cyclic AMP production curves in the presence of the two antagonists (Fig. 3), as has been described in other tissues (Laburthe et al., 1986; Pandol et al., 1986; Turner et al., 1986; Griffiths et al., 1989; Rodríguez-Pena et al., 1991); (b) the agreement between the order of efficacy of the two peptides in competition experiments (Fig. 1) with the corresponding inhibition of cyclic AMP production (Fig. 4); (c) the inefficiency of the two VIP antagonists on the stimulation of cyclic AMP production by the β-adenoceptor agonist isoproterenol (Table 2).

We have demonstrated the synergistic effect of VIP on isoproterenol-stimulated cyclic AMP production in rat and mouse peritoneal macrophages (Table 2). Thus, this VIP-mediated effect was fully abolished by 10 μM [4-Cl-D-Phe^6,Leu^{17}]VIP or 10 μM [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2 showing an interaction with a high degree of specificity. By the same sign, VIP-stimulated cyclic AMP production and the antagonist behaviour of both [4-Cl-D-Phe^6,Leu^{17}]VIP and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2 were unaffected by the adrenoceptor blockage with propranolol. Taken together, these data strongly suggest an antagonist mechanism through the VIP receptor-effector system.

The molecular characterization of antagonist effects has been performed in rat and mouse peritoneal macrophage membranes by chemical cross-linking techniques using the bifunctional reagent DTSP. After protein solubilization and SDS-PAGE, a major [^{125}I]VIP-protein complex of 52–55 kDa and 55 kDa were observed in rat and mouse peritoneal macrophage membranes, respectively. Assuming that one molecule of VIP (3.3 kDa) is linked by protein, the averages of the VIP-binding site are in the range of 50–52 kDa. Thus, the intensity of the labeling of the [^{125}I]VIP/receptor complexes was significantly lower with the antagonists than in the control experimental situations in both mouse and rat peritoneal macrophage membranes. These results are in good agreement with the binding data, with average IC_{50} values in the 100–300 nM range.

In conclusion, the studies realized with [4-Cl-D-Phe^6,Leu^{17}]VIP and [Ac-Tyr^1,D-Phe^2]GRF-(1–29)-NH_2 show that these peptides are two antagonists for the VIP receptor-effector system in rat and mouse peritoneal macrophages and their use may be a useful tool for understanding the immunomodulatory function of VIP in macrophages.

Since VIP has potent inmmunomodulatory (De La Fuente et al., 1996; Calvo et al., 1996) effects, the use of specific antagonists could be a good tool to elucidate specific and selective properties of VIP on immune function. On the other hand, the possible application of this research area to clinical immunology it is both scientifically and clinically interesting and important.

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