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Interaction of lipophilic VIP derivatives with recombinant VIP_1/PACAP and VIP_2/PACAP receptors

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Abstract

Stearyl vasoactive intestinal polypeptide has been reported to be a VIP (vasoactive intestinal polypeptide) receptor agonist of high potency with an original bioavailability and action. We synthesized three fatty acyl derivatives, myristyl-, palmityl- and stearyl-[Nle^{17}]VIP, and tested their capacity to recognize recombinant rat- and human VIP_1- and VIP_2/PACAP (pituitary adenylate cyclase-activating polypeptide) receptors and to stimulate adenylate cyclase activity. The three lipophilic analogues bound with high affinity (from 0.5 to 20 nM) to both receptor subtypes but did not distinguish between them. In preparations expressing a high density of human VIP_1/PACAP receptors, the three lipophilic analogues had the same efficacy as VIP and [Nle^{17}]VIP. In preparations expressing the rat receptors, stearyl-[Nle^{17}]VIP had a lower efficacy than the other peptides tested. In preparations expressing a low level of VIP_1/PACAP receptors and in those expressing VIP_2/PACAP receptors, all analogues behaved like partial agonists. The lowest efficacy was observed for stearyl-[Nle^{17}]VIP on the VIP_2/PACAP receptor subclass. Based on our results, a complex pattern of in vivo biological effects of the lipophilic VIP derivatives should be expected: these compounds might behave as full agonists, partial agonists, or antagonists of the VIP response, depending on the number and the subtype of receptor expressed.

Keywords: VIP (vasoactive intestinal peptide) analog; VIP_1/PACAP receptor; VIP_2/PACAP receptor

1. Introduction

Vasoactive intestinal polypeptide (VIP) is a potential therapeutic agent for several diseases—including inter-alia bronchial asthma (O'Donnell et al., 1994a,b), sexual impotence (Andersson et al., 1984; Ottesen et al., 1984), brain damage due to excitotoxic cell death (Gressens et al., 1993, 1997) and gastrointestinal motility disorders (Biancani et al., 1988).

However, several problems must be solved before it can be developed clinically: (a) VIP receptors are rather ubiquitous and numerous undesired effects are predictable. This problem might be partly surmountable, thanks to the discovery of selective agonists and antagonists (Gourlet et al., 1997a,b,c) for the two receptor subclasses expressed in different tissues (Ishihara et al., 1992; Usdin et al., 1994; Vertongen et al., 1997); (b) VIP has a rather short biological half-life (Nau et al., 1987) due to its degradation by several peptidases including aminopeptidases, neutral endopeptidase (Goetzl et al., 1989; Gourlet et al., 1997d), chymase and tryptase (Caughey et al., 1988; Franconi et al., 1989) and not yet identified peptidases present in the pulmonary alveolar fluid (Bolin et al., 1995). Stable analogues that retain a high bioactivity have already been described (O'Donnell et al., 1994a,b) but at present, it is not possible to design protease-resistant VIP analogues; (c) the bioavailability and tissue distribution of exogenously administered VIP is poorly understood but a 28-amino acid peptide is not assumed to cross easily the blood-brain barrier.

Recently, lipophilic VIP derivatives were prepared by Dr. Gozes' group (Gozes and Fridkin, 1992; Gozes et al., 1995) and were described as being superactive in in vivo and in vitro experiments. To our knowledge, these analogues have, however, not yet been quantitatively evaluated for their interaction with the two vasoactive intestinal peptide/pituitary adenylate cyclase-activating polypeptide

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(VIP/PACAP) receptor subclasses. In the present work, three lipophilic VIP derivatives (myristyl-, palmityl- and stearyl-[Nle^{17}]VIP) were synthesized and tested on rat- and human recombinant VIP_1/PACAP and VIP_2/PACAP receptors expressed in Chinese hamster ovary (CHO) cells.

2. Materials and methods

2.1. Cell lines

Six CHO cell lines expressing recombinant receptors were used in this work. The two cell lines expressing a high density (800 ± 100 fmol receptors/mg membrane protein) and a low density (90 ± 50 fmol receptors/mg membrane protein) of rat VIP_1/PACAP receptors and one cell line expressing human VIP_2/PACAP receptors (200 ± 60 fmol receptors/mg membrane protein) were previously described (Ciccarelli et al., 1994; Svoboda et al., 1994). The cell line expressing the rat VIP_2/PACAP receptor (500 ± 100 fmol receptors/mg membrane protein) was kindly provided by Dr. E.M. Lutz from the MRC Brain Metabolism Unit, Edinburgh (Lutz et al., 1993); the two cell lines expressing a high density (800 ± 100 fmol receptors/mg membrane protein—clone 25) and a low density (90 ± 50 fmol receptors/mg membrane protein—clone 9) of the human VIP_1/PACAP receptor were established by electroporation transfection of the cDNA cloned in pcDNA-3 plasmid (Invitrogen) and selection by subcloning. The clone 25 has already been used in previous studies (Gourlet et al., 1997a,b).

2.2. Membrane preparation and receptor identification

Transfected CHO cells were harvested with a rubber policeman and pelleted by low-speed centrifugation; the supernatant was discarded and the cells were lysed in 1 mM $NaHC0_3$ solution and immediately frozen in liquid nitrogen.

After thawing, the lysate was first centrifuged at 4° for 10 min at $400 \times g$ and the supernatant was further centrifuged at $20\,000 \times g$ for 10 min. The pellet, resuspended in 1 mM $NaHCO_3$, was used immediately as a crude membrane fraction.

Binding was performed as described (Ciccarelli et al., 1994; Vertongen et al., 1997), using [^{125}I]VIP for characterization of the rat and human VIP_1 receptor, and [^{125}I]Ro 25-1553 for characterization of rat and human VIP_2 receptors. Ro 25-1553 (Ac^1–[Glu^8, Lys^{12}, Nle^{17}, Ala^{19}, Asp^{25}, Leu^{26}, Lys^{27,28}, Gly^{29,30}, Thr^{31}]–NH_2 VIP(cyclo 21–25)) is VIP_2 receptor-selective and has a higher affinity than VIP for rat- and human VIP_2 receptors (Gourlet et al., 1997c). We previously radioiodinated this peptide and studied its binding to VIP_2 receptors (Vertongen et al., 1997). [^{125}I]Ro 25-1553 has a 3-fold higher affinity than [^{125}I]VIP for VIP_2/PACAP receptors, so that the results obtained with this tracer are technically more satisfactory. The EC_{50} values for unlabelled peptides are identical when [^{125}I]Ro 25-1553 or [^{125}I]VIP are used as tracers (Vertongen et al., 1997). In all cases, non-specific binding is defined as the residual binding in the presence of 1 μM VIP. Binding was performed at 37°C in a 20 mM Tris–maleate, 2 mM $MgCl_2$, 0.1 mg/ml bacitracin, 1% bovine serum albumin (pH 7.4) buffer. Bound radioactivity was separated from free radioactivity by filtration through glass-fibre GF/C filters presoaked for 24 h in 0.01% polyethyleneimine and rinsed three times with a 20-mM (pH 7.4) sodium phosphate buffer containing 1% bovine serum albumin. Adenylate cyclase activity was determined by the procedure of Salomon et al. (1974). Membrane protein (3–15 μg) was incubated in a total volume of 60 μl containing 0.5 mM [α-^{32}P]ATP, 10 μM GTP, 5 mM $MgCl_2$, 0.5 mM EGTA, 1 mM cAMP, 1 mM theophylline, 10 mM phospho(enol)pyruvate, 30 μg/ml pyruvate kinase and 30 mM Tris–HCl at a final pH of 7.5. The reaction was initiated by addition of membranes and was terminated after a 15-min incubation at 37°C by addition of 0.5 ml of a 0.5% sodium dodecylsulfate solution containing 0.5 mM ATP, 0.5 mM cAMP and 20 000 cpm [8-^{3}H]cAMP. The cAMP was separated from ATP by two successive chromatographies on Dowex 50 $W \times 8$ and neutral alumina.

2.3. Peptides synthesis

The peptides were synthesized as C-terminal amides by solid-phase methodology on an automatic Applied Biosystems apparatus (Foster City, CA, USA) using the 9-fluorenylmethoxy carbonyl strategy (Ambrosius et al., 1989). Myristic, palmitic or stearic acid was coupled to the amino terminus as 1-hydroxybenzotriazole derivative. The peptides were purified by reversed-phase chromatography on Jordi-Gel DVB 300 Å (10×1 cm) and by ion exchange chromatography on Mono S HR 5/5. Peptide purity (> 95%) was assessed by capillary electrophoresis and the sequence conformity was verified by sequencing and electrospray mass spectrometry.

For evaluation of the biological activity, 2.4×10^{-4} M stock solutions of the peptides were prepared in 50% dimethyl sulfoxide (DMSO). Further dilutions were made in water so that the final DMSO concentrations were lower than 0.2% and did not influence the binding data and basal and stimulated adenylate cyclase activity (data not shown).

2.4. Data analysis

All competition curves and dose–effect curves were analysed by anon-linear regression program (Ligand). The standard errors for the IC_{50} and EC_{50} values (on a logarithmic scale) were always less than 0.1 log unit. Differences between mean IC_{50}, EC_{50} and maximal efficacy

Table 1
The IC_{50} values (in nM) for binding of VIP, [Nle^{17}]VIP, myristyl-[Nle^{17}]VIP, palmityl-[Nle^{17}]VIP, and stearyl-[Nle^{17}]VIP at rat and human VIP_1/PACAP and VIP_2/PACAP receptors

Peptide tested	IC_{50} (nM)			
	VIP_1/PACAP R	VIP_2/PACAP R	VIP_1/PACAP R	VIP_2/PACAP R
	Rat	Rat	Human	Human
VIP	2	10	2	10
[Nle^{17}]VIP	2	8	2	3
Myristyl-[Nle^{17}]VIP	5	3	5	0.5
Palmityl-[Nle^{17}]VIP	20	10	20	3
Stearyl-[Nle^{17}]VIP	2	3	5	1

The IC_{50} values were established using the Ligand program from inhibition of tracer binding by increasing concentrations of unlabelled peptides. The results are the means of three determinations and the standard deviation was less than 0.1 log unit in all cases.

values were tested for statistical significance by Student's *t*-test; $P < 0.05$ was accepted as being significant.

3. Results

3.1. Binding properties of VIP, [Nle^{17}]VIP, myristyl-palmityl- and stearyl-[Nle^{17}]VIP

The VIP and [Nle^{17}]VIP inhibited the binding of [^{125}I]VIP or [^{125}I]Ro 25-1553 to all the cell membranes studied. The three lipophilic derivatives also inhibited tracer binding. Identical IC_{50} values (concentrations required for half-maximal binding inhibition) were obtained after 15, 30 and 45 min incubation at 37°C (data not shown), suggesting that the time necessary to reach equilibrium was not different for the tracers and the competitors (Motulsky and Mahan, 1984). The IC_{50} values are summarized in Table 1. Myristyl-[Nle^{17}]VIP and stearyl-[Nle^{17}]VIP had lower IC_{50} values than [Nle^{17}]VIP at the VIP_2/PACAP receptors but not at the VIP_1/PACAP receptors ($P < 0.05$). Palmityl-[Nle^{17}]VIP had a higher IC_{50}

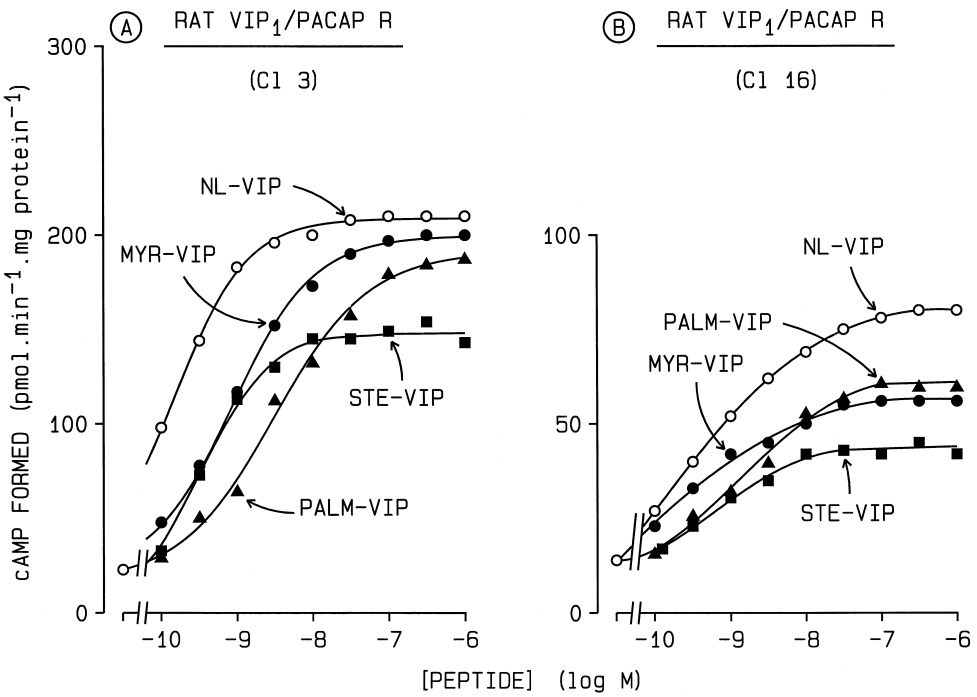

Fig. 1. Dose–effect curves of [Nle^{17}]VIP (○), myristyl-[Nle^{17}]VIP (MYR-VIP) (●), palmityl-[Nle^{17}]VIP (PALM-VIP) (▲), and stearyl-[Nle^{17}]VIP (STE-VIP) (■) on adenylate cyclase activation from CHO cells expressing a high density (Cl 3, left panel) or a low density (Cl 16, right panel) of rat VIP_1/PACAP receptors. The results are expressed in pmol cyclic AMP produced per minute milligram of protein and are representative of three experiments. The [Nle^{17}]VIP EC_{50} value was lower for Cl 3 (0.15 nM) than Cl 16 (0.5 nM) membranes, but the EC_{50} values for myristyl-[Nle^{17}]VIP (1.0 nM), palmityl-[Nle^{17}]VIP (3.0 nM) and stearyl-[Nle^{17}]VIP (0.5 nM) were identical for membranes from both clones. Stearyl-[Nle^{17}]VIP was a partial agonist ($P < 0.05$) in both clones. Myristyl-[Nle^{17}]VIP and palmityl-[Nle^{17}]VIP induced a maximal adenylate cyclase activation (using [Nle^{17}]VIP as reference) on Cl 3 ($P > 0.05$) but were partial agonists on Cl 16 ($P < 0.05$).

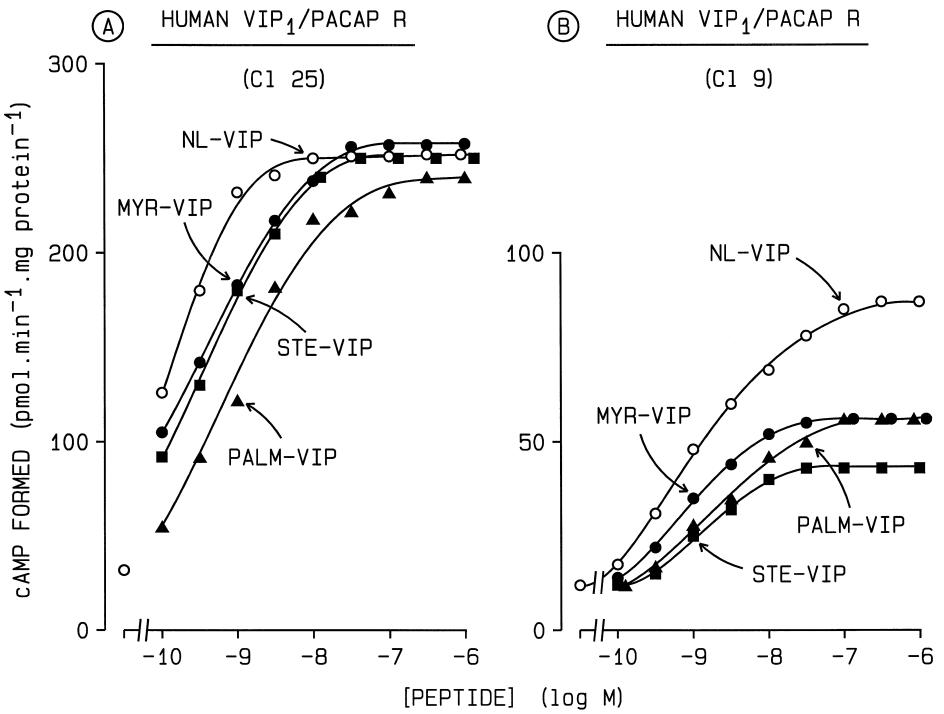

Fig. 2. Same experimental design, symbols and expression of the results as in Fig. 1, but the study was performed with membranes from CHO cells expressing a high density (Cl 25, left panel) or a low density (Cl 9, right panel) of the human VIP_1/PACAP recombinant receptor. The EC_{50} values for [Nle^{17}]VIP, myristyl-[Nle^{17}]VIP, palmityl-[Nle^{17}]VIP and stearyl-[Nle^{17}]VIP were lower for Cl 25 membranes (0.15, 0.30, 1.0 and 0.30 nM, respectively) than for Cl 9 membranes (1.0, 1.0, 2.0 and 1.0 nM, respectively). Myristyl-[Nle^{17}]VIP, palmityl-[Nle^{17}]VIP and stearyl-[Nle^{17}]VIP were able to activate maximally the adenylate cyclase in Cl 25 (using [Nle^{17}]VIP as reference) but behaved as partial agonists ($P < 0.05$) on Cl 9 membranes.

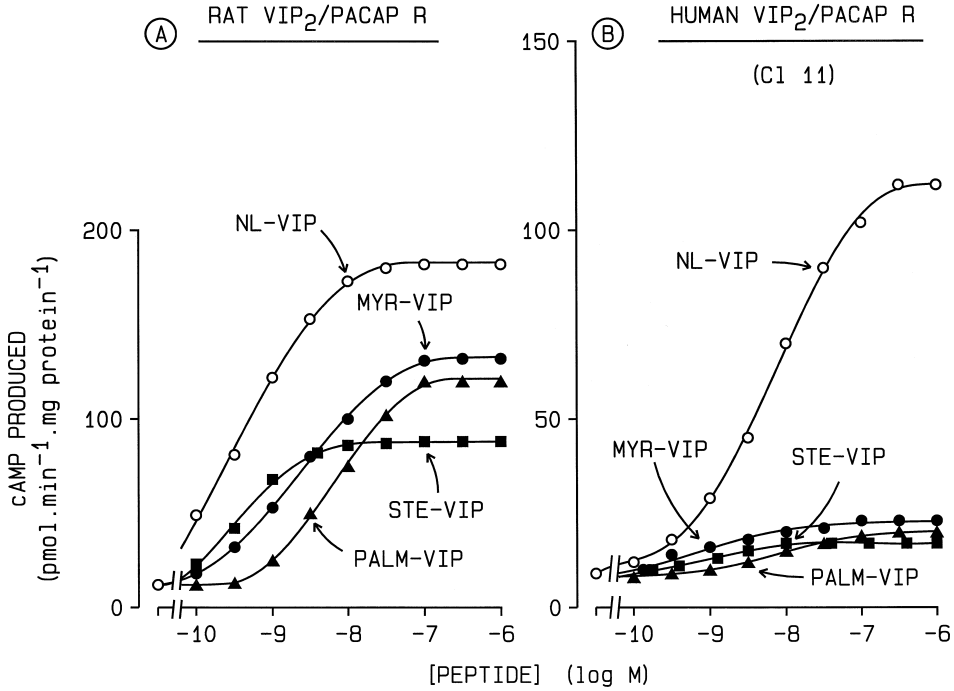

Fig. 3. Same experimental design, symbols and expression of the results as in Figs. 1 and 2. The study was performed with membranes from CHO cells expressing the rat- (left panel) and human (right panel) VIP_2/PACAP receptors. [Nle^{17}]VIP, myristyl-[Nle^{17}]VIP, palmityl-[Nle^{17}]VIP and stearyl-[Nle^{17}]VIP activated the adenylate cyclase via rat VIP_2/PACAP receptors with EC_{50} values of 0.3, 3.0, 7.0 and 0.5 nM, respectively. The three lipidic VIP analogues were partial agonists at rat- and human VIP_2 receptors ($P < 0.05$).

value at rat and human VIP_1/PACAP receptors ($P < 0.05$) and was equipotent to [Nle^{17}]VIP at the VIP_2/PACAP receptor.

3.2. Effects of VIP, [Nle^{17}]VIP and the lipophilic analogues on adenylate cyclase activation

In all the systems tested, VIP and [Nle^{17}]VIP were equally potent and efficient (data not shown). When tested on cell membranes that expressed a high density of rat- (Fig. 1, left panel) or human (Fig. 2, left panel) VIP_1/PACAP receptors, palmityl-[Nle^{17}]VIP and myristyl-[Nle^{17}]VIP were less potent but as efficient as the [Nle^{17}]VIP standard. Stearyl-[Nle^{17}]VIP was less efficient than the other molecules tested at the rat (but not human) VIP_1/PACAP receptor. When tested in clones expressing lower receptor densities, the myristyl-, palmityl- and stearyl-[Nle^{17}]VIP had significantly lower efficacies (Fig. 1, right panel; Fig. 2, right panel). The lowest efficacy was observed for the stearyl analogue.

On cell membranes that expressed the recombinant rat VIP_2/PACAP receptor (Fig. 3, left panel), myristyl- and palmityl [Nle^{17}]VIP had a reduced potency, and stearyl-[Nle^{17}]VIP was equipotent to [Nle^{17}]VIP (Fig. 3). The three analogues had a reduced efficacy. As for VIP_1/PACAP receptors, stearyl-[Nle^{17}]VIP had the lowest efficacy.

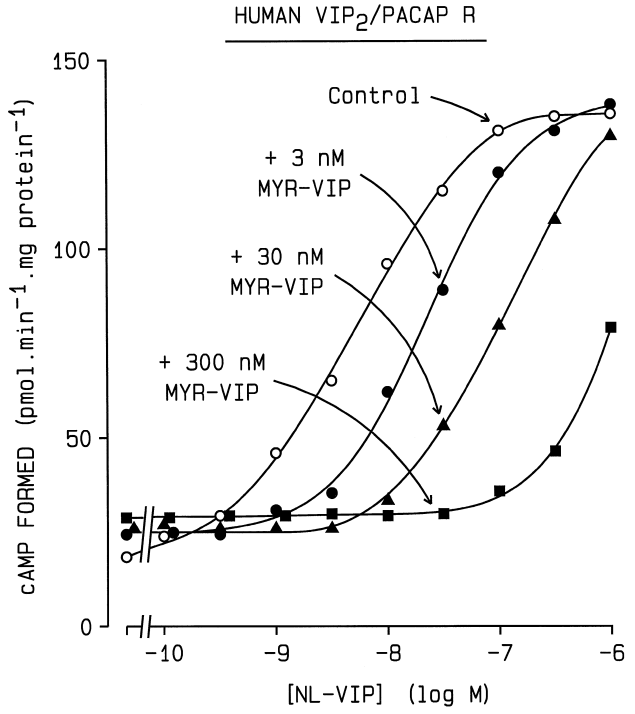

Fig. 4. Dose–effect curves of [Nle^{17}]VIP-stimulated adenylate cyclase activity on CHO cell membranes expressing the human VIP_2/PACAP receptor in absence (○) or presence of 3 nM (●), 30 nM (▲) and 300 nM myristyl-[Nle^{17}]VIP (■). One experience representative of three others. The [Nle^{17}]VIP EC_{50} value increased significantly ($P < 0.05$), from 5 nM to 20, 100 and 1000 nM in the presence of 3, 30 and 300 nM myristyl-[Nle^{17}]VIP, respectively.

On membrane preparations expressing the recombinant human VIP_2/PACAP receptor (Fig. 3, right panel), the intrinsic activity of myristyl-, palmityl- and stearyl-[Nle^{17}]VIP was low (between 0.1 and 0.2, considering the maximal effect of [Nle^{17}]VIP as 1.0) and these analogues were thus clearly partial agonists. This was further demonstrated by the rightward shift of the dose–response curve of [Nle^{17}]VIP in the presence of increasing concentrations of myristyl-[Nle^{17}]VIP (Fig. 4). From these data, a K_i value of 2 nM was calculated for myristyl-[Nle^{17}]VIP and 10 nM and 1 nM for palmityl- and stearyl-[Nle^{17}]VIP analogues (data not shown), respectively.

4. Discussion

Gozes and Fridkin (1992) developed a strategy to design VIP derivatives with an increased hydrophobicity by conjugating VIP with stearic acid by an amide bond at the amino terminus of the peptide chain. The stearyl VIP was as active as VIP on rat sexual function when administered intravenously and more potent when administered topically. A second derivative in which the methionine 17 residue was replaced by a norleucine residue was even more potent in the same model (Gozes et al., 1994). Stearyl-[Nle^{17}]VIP was also found to be 100-fold more potent than VIP in promoting neuronal cell survival in dissociated spinal cord cells (Gozes et al., 1995).

In the present study, we synthesized three [Nle^{17}]VIP derivatives with a fatty acyl moiety of 14, 16 and 18 carbons and compared their binding properties and their capacity to stimulate adenylate cyclase activity in CHO cells expressing rat- and human recombinant wild-type VIP_1/PACAP and VIP_2/PACAP receptors.

Our major findings were: (1) the three lipophilic derivatives recognized both receptor subclasses with an affinity comparable to that of [Nle^{17}]VIP and did not display a significant selectivity for one of the two VIP receptor subclasses; (2) the presence of a free amino terminus function was not necessary for high-affinity receptor recognition, confirming previous data obtained with acetyl-[His^1]VIP (Gourlet et al., 1991); (3) myristyl-, palmityl- and stearyl-[Nle^{17}]VIP were less efficient than [Nle^{17}]VIP and VIP on adenylate cyclase activation and were thus partial agonists or ‘dualists’. This effect was particularly evident in cell lines expressing rat- and human VIP_2 receptors (Fig. 3) and in cell lines expressing a low density of VIP_1 receptors (rat VIP_1 clone 16 and human VIP_1 clone 9: Figs. 1 and 2). The intrinsic activity of the lipophilic derivatives at VIP_1 receptors depended in part on the receptor density. It was higher in cell lines expressing high receptor densities (rat VIP_1 clone 3 and human VIP_1 clone 25: Figs. 1 and 2). Stearyl-[Nle^{17}]VIP might have a lower intrinsic activity at rat VIP_1 receptors, as it behaved as a partial agonist even in the cell line (rat VIP_1 clone 3) expressing a high receptor density (Fig. 1).

The partial agonists of VIP/PACAP receptors described so far are VIP analogues or fragments modified in the amino terminal moiety, such as VIP-(2–28) and VIP-(3–28) (Ciccarelli et al., 1994) or [D-Phe^2]VIP, [D-Arg^2]VIP, [D-His^1]VIP (Robberecht et al., 1986). This suggests that a proper positioning and/or a precise structure of the amino terminal part of the molecule is crucial to induce the active receptor conformation. The hydrophobic chain might interact with the lipid bilayer, with a receptor exosite (like the lipophilic β-adrenoceptor agonist, salmeterol) (Jack, 1997) or with the core of the VIP peptide, on the hydrophobic face of the amphiphilic helix (Musso et al., 1988).

It is difficult to predict the biological effects of partial agonists—both in vitro and in vivo. Indeed, their efficacy is qualitatively and quantitatively dependent on the cell or tissue considered, because it varies with the number of receptors expressed and the coupling efficacy of the occupied receptors to the second messenger systems.

It has been shown (Gozes et al., 1995) that stearyl-[Nle^{17}]VIP increased the survival of cultured murine neuroblast under conditions where there was no detectable increase in cyclic AMP levels. This was attributed to the presence on these cells of a not yet cloned VIP receptor. The present findings offer alternative explanations: neuroblasts might express VIP_2/PACAP receptors (with a high affinity for the lipidic analogues) poorly coupled to the G_s proteins and therefore unable to increase cAMP in response to stearyl-[Nle^{17}]VIP. The lipidic analogue might also favour VIP_2/PACAP receptor coupling to other G proteins: we previously observed that VIP receptors may be coupled to G_i proteins (van Rampelbergh et al., 1997) and the receptor-G protein coupling efficiency may depend on the agonist considered (Spengler et al., 1993).

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