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Properties of the Pituitary Adenylate Cyclase-Activating Polypeptide I and II Receptors, Vasoactive Intestinal Peptide$_1$, and Chimeric Amino-Terminal Pituitary Adenylate Cyclase-Activating Polypeptide/Vasoactive Intestinal Peptide$_1$ Receptors: Evidence for Multiple Receptor States

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SUMMARY

We analyzed the functional and binding properties of the "normal" pituitary adenylate cyclase-activating polypeptide (N-PACAP) type I, PACAP type II/vasoactive intestinal peptide (VIP)$_1$, and chimeric N-PACAP/VIP$_1$ receptors expressed in Chinese hamster ovary cells. The binding properties of the three receptors were investigated using three radioiodinated tracers: ^{125}I-VIP, ^{125}I-PACAP-27, and ^{125}I-PACAP-29 (^{125}I-PACAP-27-Gly28,Lys29-amide). The three tracers labeled very different receptor densities; ^{125}I-PACAP-29 labeled more receptors than either ^{125}I-VIP or ^{125}I-PACAP-27 in the three cell lines. Analysis of the competition curves suggested that the three tracers labeled in a different manner three PACAP I receptor states, two PACAP II/VIP$_1$ receptor states, and three chimeric N-PACAP/VIP$_1$ receptor states in transfected Chinese hamster ovary cells. The previously described PACAP$_{1A}$ and PACAP$_{1B}$ receptors, which differ by their affinities for PACAP-27 and PACAP-38, actually correspond to different PACAP I receptor states. The three receptors were able to increase adenylate cyclase activity when activated by PACAP-38, PACAP-27, or VIP. In contrast with the two parent receptors, the chimeric N-PACAP/VIP$_1$ receptor was activated by PACAP-38 at lower concentrations than PACAP-27, suggesting that the amino-terminal and core receptor domains influence each other and that the conformation of one or both domains was altered in the chimeric compared with wild-type receptors. Comparison of the binding and functional properties of three clones expressing different chimeric N-PACAP/VIP$_1$ receptors densities indicated that ^{125}I-PACAP-29 was necessary to correctly estimate the receptor number and that ^{125}I-PACAP-27 or ^{125}I-VIP labeled only a fraction of the functional receptors. We suspect (but could not demonstrate) that this might also be true for PACAP I and PACAP II/VIP$_1$ receptors.

Three receptors for VIP and PACAP have been cloned (1–4); they belong to the family of G protein-coupled receptors. The PACAP I receptors are highly selective for PACAP. The PACAP II/VIP$_1$ and PACAP II/VIP$_2$ receptors do not discriminate PACAP from VIP (5) but have different pharmacological profiles (6–8) for PACAP and VIP analogs. Five variants resulting from alternative splicing were identified for the PACAP I receptors (9); they differ in the length of the third intracellular loop connecting transmembrane helices V and VI.

PACAP I and PACAP II/VIP$_1$ receptors recognize with the same high affinity (2–4 nM) the two molecular forms of PACAP (the short form of 27 amino acids and the long form of 38 amino acids) but recognize VIP in a different manner: the K_D values for VIP on PACAP I and PACAP II/VIP$_1$ receptors were 1000 and 2 nM, respectively (5). Because the largest differences in amino acid sequence of the two receptors are seen in the amino-terminal portion of the receptors, we suggested that this part of the PACAP I receptor might be responsible for the increased selectivity of the receptor. To test this hypothesis, we constructed a chimeric receptor consisting of the amino-terminal extracellular domain of the PACAP I receptor and the core of the PACAP II/VIP$_1$ receptor. The construction was transfected in CHO cells, which do not express constitutively PACAP I or PACAP II receptors, and clones expressing the receptor protein were selected. Their coupling to adenylate cyclase was compared with that of the wild-type PACAP I and PACAP II/VIP$_1$ recombinant receptors (10, 11).

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ABBREVIATIONS: PACAP, pituitary adenylate cyclase activating polypeptide; VIP, vasoactive intestinal peptide; CHO, Chinese hamster ovary.

Several antagonists that recognize the three PACAP receptor subtypes have been identified. However, they have a low affinity for the receptors and therefore cannot be used as radioligands for binding studies. The only available radioligands for this receptor family are agonists (i.e., ligands that are likely to prefer the receptor/G protein complex to the "uncoupled" receptor).

Two natural forms of PACAP are synthesized through alternative processing of the peptide precursor: PACAP-27 and the peptide PACAP-38, which has a prolonged carboxyl terminus. We previously observed in tissues and neuroblastoma cells expressing PACAP I receptors that ^{125}I-PACAP-38 labeled the same receptor population as ^{125}I-PACAP-27 as well as an additional receptor population. We called the receptors that did not discriminate PACAP-27 and PACAP-38 A receptors, and we called the PACAP-38-preferring receptors B receptors (12, 13).

^{125}I-PACAP-38 binding studies are extremely difficult to perform, due in part to the high nonspecific interaction of this radioligand with the filters, plasticware, and so on. We previously synthesized several PACAP-38 fragments of different lengths and observed that PACAP-29 (PACAP-27-Gly28,Lys29-amide) was the shortest peptide with the same pharmacological profile as PACAP-38 (14). We therefore decided to radioiodinate PACAP-29 and compare its binding properties with the binding properties of ^{125}I-PACAP-27 and ^{125}I-VIP to the PACAP I receptors, PACAP II/VIP$_1$ receptors, and chimeric N-PACAP/VIP$_1$ receptors. The comparison of the binding properties of ^{125}I-PACAP-27 and ^{125}I-PACAP-29 with the adenylate cyclase stimulation in several clones with different receptor densities led us to suggest that in contrast to ^{125}I-PACAP-27, ^{125}I-PACAP-29 is able to recognize all of the PACAP receptors, whether coupled or not coupled to the G protein.

Materials and Methods

Construction of the expression plasmid, transfection, selection, and expression in CHO cells. The products used for recombinant DNA expression and cell cultures as well as the procedure used to obtain CHO cell lines that express the wild-type PACAP I and PACAP II/VIP$_1$ rat receptors have been described previously (10, 11). The chimeric receptor consisting in the amino-terminal part of the PACAP I receptor and the core of the PACAP II/VIP$_1$ receptor (N-PACAP/VIP.r) was generated through ligation of three cDNA fragments: (i) the PACAP I receptor cDNA fragment obtained by the action of *Hin*dIII and *Bsl*I that corresponded to amino acids 1–141 plus a portion of the 5′-untranslated region, (ii) 36-bp *Bsl*I/*Cfr*10I double-stranded synthetic adapter synthesized according to the solid-phase phosphoramidate method, and (iii) the VIP$_1$ receptor *Cfr*10I/*Not*I cDNA fragment coding for the portion of the receptor from the first amino acid of transmembrane I to the stop codon.

The N-PACAP/VIP.r chimera was inserted in the plasmid pBluescript SK$^+$ (Stratagene, La Jolla, CA) previously digested with *Hin*dIII/*Not*I. The *Hin*dIII/*Not*I coding cassette was excised and introduced into the mammalian expression vector pCDNA3 (InVitrogen, San Diego, CA). The sequence of the construction was verified by direct sequencing. The plasmid was transfected into the CHO cell line DG44 by electroporation using a gene pulser. Approximately 10^7 cells were preincubated on ice for 30 min with 25 μg of DNA in 0.8 ml of 7 mM sodium phosphate buffer, pH 7.4, containing 272 mM sucrose and 1 mM $MgCl_2$. Electroporation was performed at 600 V and 3 μF. Cells were maintained in α-minimal essential medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin with an atmosphere of 95% air/5% CO_2 at 37°. At 48 hr after transfection, geneticin (400 μg/ml) was added to the culture medium, and geneticin-resistant cells were selected. Clones were obtained through cell dilution, and the final selection was made based on their ability to bind ^{125}I-PACAP-27 and to express a PACAP-stimulated adenylate cyclase activity. The study was conducted on three clones (clones 2, 11, and 22) that expressed different receptor densities. Using reverse-transcription polymerase chain reaction with the appropriate primers, we verified that each cell line exclusively expressed the expected mRNA. The CHO cells were routinely grown in Dulbecco's medium enriched with 10% fetal calf serum, and geneticin was maintained in the stock cultures only.

Membrane preparation, receptor identification, and adenylate cyclase activity. 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 $NaHCO_3$ solution followed by immediate freezing in liquid nitrogen. After thawing, the lysate was first centrifuged at 800 × g for 10 min; then, the supernatant was further centrifuged at 20,000 × g for 10 min. The pellet, resuspended in 1 mM $NaHCO_3$ was used immediately as a crude membrane preparation. Like ^{125}I-VIP and ^{125}I-PACAP-27 (1.0 mCi/nmol), ^{125}I-PACAP-29 was labeled by the iodogen method as described previously (15), purified by adsorption on a Sep-Pak cartridge (Waters, Milford, MA), and eluted with 50% acetonitrile in 0.1% trifluoroacetic acid. To check the purity of the tracers, a sample of the Sep-Pak eluate was analyzed by high performance liquid chromatography (15). The elution profiles of the three tracers were very similar. Tracer binding to the membranes was performed as described previously (15). The incubation was for 20 min at 37° in a 20 mM Tris·HCl buffer, pH 7.4, enriched with 5 mM $MgCl_2$, 0.5 mg/ml bacitracin, 100 kallikrein-inhibitory units/ml of Trasylol, 1% (w/v) bovine serum albumin, 20,000 cpm/assay radioligand (corresponding to 0.10 nM tracer), and CHO cell membranes at a concentration sufficient to bind 10–15% of the tracer offered. Tracer binding was determined after filtration through glass-fiber filters (Gelman A/C, Gelman Sciences, Ann Arbor, MI) soaked overnight in 0.1% polyethyleneimine (Sigma Chemical, St Louis, MO). Adenylate cyclase activity was determined according to the method of Salomon *et al.* (16) as described previously (14, 15).

Mathematical analysis of the competition curves. We observed as a rule that the number of labeled receptors and their apparent affinities for the unlabeled peptides varied markedly depending on the tracer chosen (see Results). We suspected that this might reflect the binding profile of the peptides: a peptide with a micromolar K_D value for a given receptor state is unlikely to label this receptor. We therefore decided to analyze simultaneously the three competition curves obtained with the two or three tracers in each membrane preparation. The curve fitting was significantly improved when we assumed that two PACAP II/VIP$_1$ and three PACAP I and chimeric N-PACAP/VIP$_1$ receptor states coexist in the membranes of the cloned CHO cells. The competition curves were therefore fitted to the following equation:

$$B = \frac{B_{SH} K_{SH}}{K_{SH} + P} + \frac{B_H K_H}{K_H + P} + \frac{B_L K_L}{K_L + P}$$

where B represents the tracer binding in the presence of the unlabeled peptide; B_{SH}, B_H, and B_L represent the percentage of bound tracer labeling the super high, high, and low, respectively, affinity states in the absence of peptide; K_{SH}, K_H, and K_L represent the dissociation constants of the unlabeled peptide for each receptor state, and P represents the unlabeled peptide concentration.

B_{SH}, B_H, and B_L depend on the density of each receptor state as well as the affinity of the tracers for these states. We therefore used these parameters to fit the three unlabeled peptide competition curves obtained with each tracer. On the other hand, because the values of the dissociation constants K_{SH}, K_H, and K_L are intrinsic properties of the unlabeled peptides, we used these constants when

• fitting the competition curves obtained with the three (or two) tracers, using each unlabeled peptide.

Peptide synthesis. All of the peptides used (Table 1) were synthesized in the laboratory by A. Vandermeers, M.-C. Vandermeers-Piret, and P. Gourlet using the solid-phase methodology. PACAP-29 (PACAP-27-Gly28,Lys29-amide) was synthesized as a carboxyl-terminal amide (14).

Results

Adenylate cyclase stimulation by the different peptides. We previously described the adenylate cyclase stimulation pattern observed in membranes of CHO cells expressing the normal PACAP I receptor [P2-10 cells (11)] and the PACAP II/VIP_1 receptor [VIP_1, clone 3 cells (10)]. The EC_{50} values obtained in the different cell lines are summarized in Table 2.

We previously observed that amino-terminal truncated PACAP-38 fragments had a very different receptor recognition profile than the corresponding PACAP-27 fragments (15). We also observed that two additional carboxyl-terminal amino acids were sufficient to confer PACAP-38-like properties to the PACAP-27 fragments (14). We therefore decided to compare the functional properties of PACAP-27, PACAP-29 (14), and PACAP-38 in the different cell lines. The EC_{50} values are summarized in Table 2, and the adenylate cyclase stimulation curves are shown in Fig. 1.

We investigated tracer binding to PACAP II/VIP_1 receptors (VIP_1 clone 3 cell membranes), PACAP I receptors (P2-10 cell membranes), and chimeric N-PACAP/VIP_1 receptors (PV clone 11 cell membranes).

Kinetics. We attempted to label the receptors using three different radioiodinated peptides (^{125}I-VIP, ^{125}I-PACAP-27, and ^{125}I-PACAP-29) and compared the dissociation rates of the three tracers induced by addition of 1 μM PACAP-27 or by the simultaneous addition of PACAP-27 and GTP (100 μM). The three tracers bound rapidly and reversibly to VIP_1 receptors (data not shown). ^{125}I-VIP and ^{125}I-PACAP-27 dissociation from these receptors was rapid (half-life, 5 min) and biphasic. It was accelerated by GTP (half-life, <1 min). ^{125}I-PACAP-29 dissociation was somewhat slower (half-life, ~10 min) and less sensitive to GTP addition than ^{125}I-VIP or ^{125}I-PACAP-27 dissociation (half-life, 5 min in the presence of GTP).

We were unable to observe specific ^{125}I-VIP binding to PACAP I receptors. ^{125}I-PACAP-27 and ^{125}I-PACAP-29 labeling of this receptor subtype was rapid and reversible (data not shown). The ^{125}I-PACAP-27 dissociation rate was rapid (half-life, ~10 min), biphasic, and accelerated in the presence of GTP (half-life, ~4 min; see Ref. 11). In contrast, the ^{125}I-PACAP-29 dissociation rate was much slower (half-life, >15 min), apparently monophasic, and not significantly affected by the addition of GTP to the dissociation medium (data not shown).

The three tracers labeled chimeric N-PACAP/VIP_1 receptors (PV clone 11) rapidly and reversibly (data not shown). Dissociation of the three tracers from the chimeric receptor was faster than dissociation from the wild-type PACAP I or PACAP II/VIP_1 receptors (half-life, <1 min). The ^{125}I-VIP and ^{125}I-PACAP-27 dissociation rates were markedly accelerated by GTP addition; the ^{125}I-PACAP-29 dissociation was accelerated only 2-fold in the presence of GTP (not shown).

Effect of GTP on equilibrium tracer binding. As shown in Fig. 2, GTP markedly and dose-dependently inhibited steady state ^{125}I-VIP binding to the PACAP II/VIP_1 and to chimeric N-PACAP/VIP_1 receptors. Steady state ^{125}I-PACAP-27 binding to the three receptors was also inhibited by GTP. In contrast, ^{125}I-PACAP-29 binding to the three receptors was barely affected, if at all, by GTP addition to the incubation medium.

Determination of the receptor concentrations. In contrast with CHO cells expressing VIP_1 receptors (10), we were not able to achieve receptor saturation with the radiolabeled peptides, using membranes from CHO cells expressing the PACAP I receptors or the chimeric N-PACAP/VIP_1 receptors.

To measure the receptor concentrations, we therefore decided to analyze detailed competition curves, with the assumption that [as previously observed with naturally expressed PACAP I and VIP_1 receptors (12, 17)] the labeled and unlabeled peptides had similar binding properties for the three recombinant receptors studied in the present study (18). Analysis of the VIP competition curves on CHO cell membranes expressing VIP_1 receptors supported this hypothesis, Because the unlabeled peptide had the same affinity (K_D = 0.5 nM) as the labeled peptide for the recombinant VIP_1 receptors (current results and Ref. 10).

We prepared homologous (^{125}I-VIP/VIP, ^{125}I-PACAP-27/PACAP-27, and ^{125}I-PACAP-29/PACAP-29) competition curves with additional peptide concentrations (three instead of two concentrations per logarithm) to improve the precision of the analysis using membranes from CHO cells expressing PACAP I, PACAP II/VIP_1, or chimeric N-PACAP/VIP_1 receptors.

^{125}I-VIP binding to VIP_1 receptors has been described previously (10). This tracer did not label PACAP I receptors, and it labeled two receptor populations in CHO cell membranes expressing chimeric N-PACAP/VIP_1 receptors (data not shown). ^{125}I-PACAP-27 labeled two receptor populations in CHO cell membranes expressing VIP_1 receptors and only one receptor population in CHO cells expressing PACAP I and chimeric N-PACAP/VIP_1 receptors. ^{125}I-PACAP-29 labeled a high density of apparently homogeneous receptors in all of the clones studied. The high affinity B_{max} values are summarized in Table 3, and representative saturation curves are shown in Fig. 3.

Competition curve fitting. We analyzed the competition curves obtained with the three tracers in the three cell lines as explained in Materials and Methods, with the assumption

TABLE 1

Sequence of the peptides used in the study

Differences from PACAP are underlined.

VIP	H-S-D-A-V-F-T-D-N-Y-T-R-L-R-K-Q-M-A-V-K-K-Y-L-N-S-I-L-N-NH_2
PACAP-27	H-S-D-G-I-F-T-D-S-Y-S-R-Y-R-K-Q-M-A-V-K-K-Y-L-A-A-V-L-NH_2
PACAP-29	H-S-D-G-I-F-T-D-S-Y-S-R-Y-R-K-Q-M-A-V-K-K-Y-L-A-A-V-L-G-K-NH_2
PACAP-38	H-S-D-G-I-F-T-D-S-Y-S-R-Y-R-K-Q-M-A-V-K-K-Y-L-A-A-V-L-G-K-R-Y-Q-R-V-K-N-K-NH_2

TABLE 2

EC_{50} (in nM) values of adenylate cyclase stimulation by PACAP-27, PACAP-29, PACAP-38, and VIP in CHO cell membranes expressing PACAP I, PACAP II/VIP_1, and chimeric N-PACAP/VIP_1 receptors (clones 11, 22, and 2).[a]

	PACAP-38	PACAP-27	PACAP-29	VIP
PACAP I	0.1[b]	0.2	0.1	80
PACAP II VIP_1	0.1	0.3	0.1	0.1
PACAP/VIP clone 11	0.05	0.5	0.05	2
PACAP/VIP clone 22	0.3	3.0	0.3	7
PACAP/VIP clone 2	0.5	5.0	0.5	20

[a] The maximal stimulations were 6-, 8-, and 7.5-fold basal in clones 2, 22, and 11, respectively (not significantly different).

[b] All EC_{50} values are the average of at least three determinations performed in duplicate. The standard deviations were always <0.1 log units (±15% of the average value).

that the radioligands recognized two or three "binding sites." The proportion of bound tracer labeling each site (B_{SH}, B_H, and B_L) and the unlabeled ligand IC_{50} values (K_{SH}, K_H, and K_L) are summarized in Table 4.

The data obtained with CHO cell membranes expressing VIP_1 receptors were compatible with the existence of two binding sites, or states (a high affinity site labeled by the three tracers and a low affinity site), that were detectable in some but not all of ^{125}I-VIP binding studies, in all of ^{125}I-PACAP-27 binding studies, and most of the ^{125}I-PACAP-29 binding.

The data obtained with CHO cell membranes expressing PACAP I receptors (Fig. 4) were compatible with the existence of two PACAP-27 binding sites, or states: a high affinity site labeled by both ^{125}I-PACAP peptides and a low affinity site recognized by ^{125}I-PACAP-29 only. The high affinity site labeled by ^{125}I-PACAP-27 could be further subdivided in two states, with higher (super high sites) and lower (high sites) affinity for VIP. Three sites, or states, were therefore necessary to describe the data.

In CHO cell membranes expressing N-PACAP/VIP_1 chimeric receptors, ^{125}I-VIP clearly labeled a receptor state with very high affinity for VIP (super high receptors; Fig. 5A). A second receptor state (representing only 30% of tracer binding) with a lower affinity for VIP was also labeled by this tracer; however, because of the low proportion of tracer binding to this site, we were unable to determine the corresponding VIP IC_{50} values with precision by using this tracer (Fig. 5A). ^{125}I-PACAP-29 clearly labeled a larger receptor concentration, including a receptor state with very low affinity for VIP and PACAP-27 (Fig. 5C). Because this population of receptor represented 85% of the total radioactivity bound, the IC_{50} values could be determined unambiguously. In contrast, we were unable to determine with precision the IC_{50} values of the unlabeled peptides for the high affinity sites (15% of the total radioactivity bound). The ^{125}I-PACAP-27 competition curves, although intermediate between the ^{125}I-VIP and ^{125}I-PACAP-29 competition curves (Fig. 5B), could not be fitted by making the assumption that only two receptor states with IC_{50} values of 1 nM and 2 μM for VIP existed in these membranes: a clearly biphasic rather than shallow VIP competition curve would have been obtained if this was the case. The curve fitting was significantly improved by making the assumption that the majority of the binding sites labeled by ^{125}I-PACAP-27 had an intermediate (high) affinity for VIP (Table 4). By fixing the K_{SH} and K_L values (obtained from ^{125}I-VIP and ^{125}I-PACAP-29 competition curves, respectively), we were able to determine with a high degree of confidence the values of K_H.

We subsequently analyzed VIP, PACAP-27, PACAP-29, and PACAP-38 competition curves obtained with the three

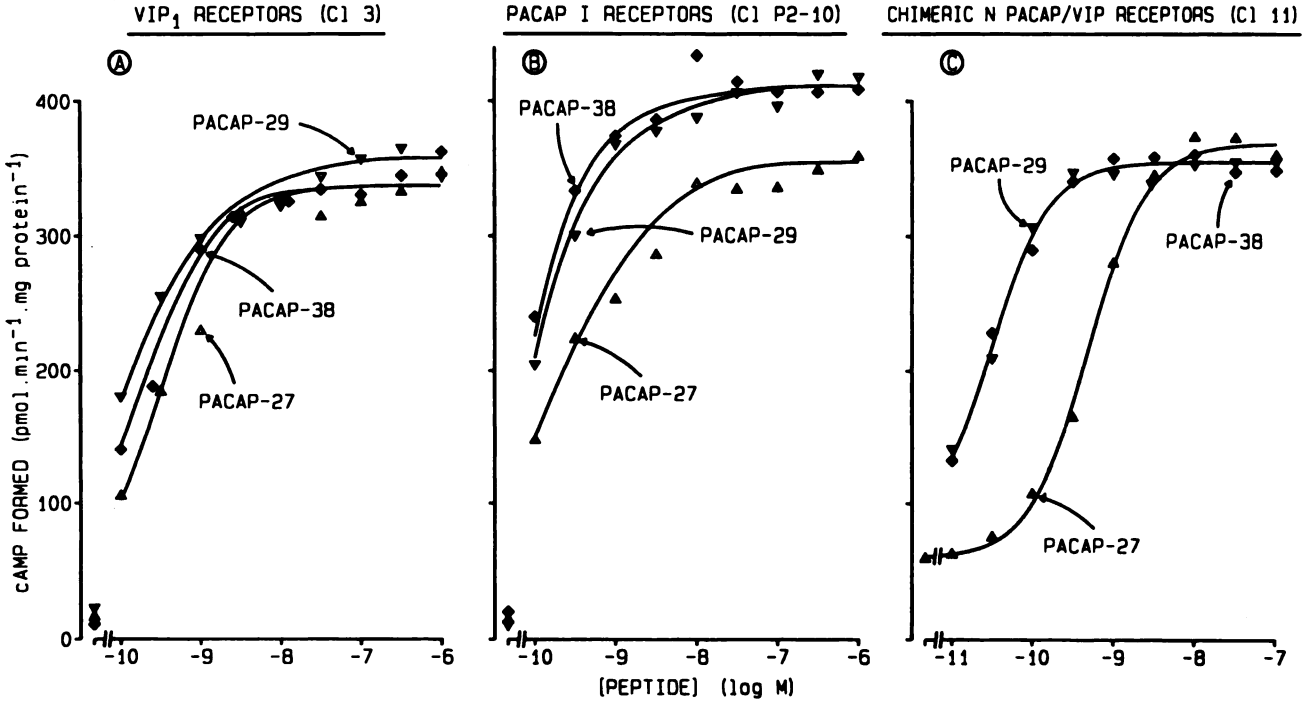

Fig. 1. Effect of increasing concentrations of (△) PACAP-27, (▽) PACAP-29, and (♦) PACAP-38 on adenylate cyclase activation of CHO cell membranes expressing (A) VIP_1 receptors (clone 3), (B) PACAP I receptors (clone P2-10), or (C) chimeric N-PACAP/VIP_1 receptors (clone 11). The results were the mean of three experiments.

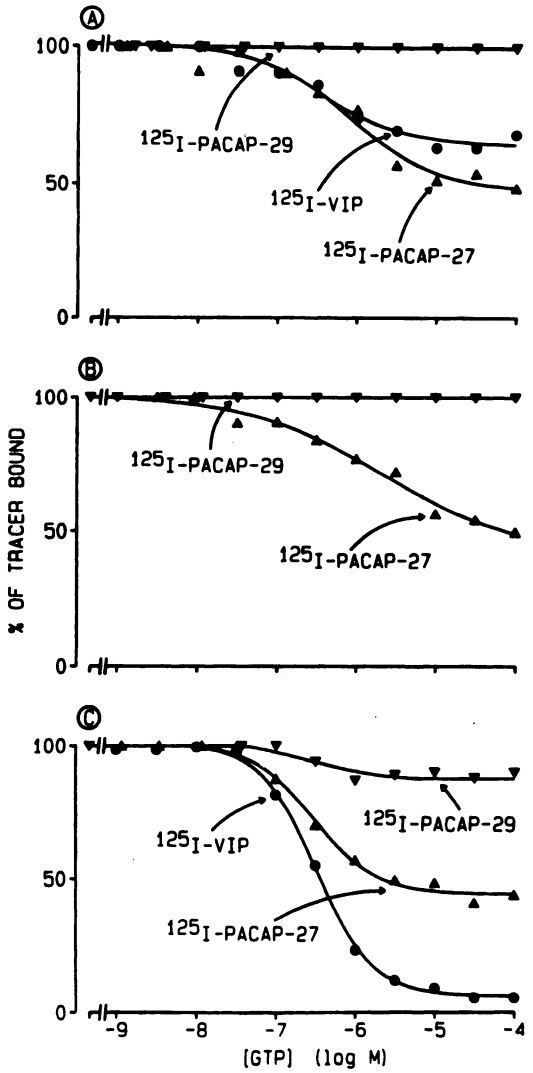

Fig. 2. Effect of GTP on equilibrium tracer binding. A, Effect of increasing GTP concentration on the specific binding of (●) ^{125}I-VIP, (▲) ^{125}I-PACAP-27, and (▼) ^{125}I-PACAP-29 to membranes of CHO cells expressing VIP_1 receptors (clone 3). B, As in A, membranes were from CHO cells expressing PACAP I receptors (clone P2-10). C, As in A, membranes were from CHO cells expressing the chimeric N-PACAP/VIP_1 receptor (clone 11). The results are expressed in percentage of tracer bound in the absence of added nucleotide and represent the average of three experiments in duplicate.

TABLE 3

Receptor concentration labeled by each radioligand

The receptor concentrations in (pmol/mg of protein) were estimated by analysis of "homologous" ^{125}I-peptide/unlabeled peptide competition curves, as explained in the text. The 95% confidence intervals are given in parentheses.

	^{125}I-VIP	^{125}I-PACAP-27	^{125}I-PACAP-29
VIP_1 receptors	5.0 (3.0–7.0)	5.0[a] (3.0–7.0)	45 (40–55)
PACAP I receptors		7.8 (6.4–9.0)	29.0 (25–32)
N-PACAP/VIP_1 receptors			
Clone 11	0.4[a] (0.2–0.6)	2.8 (2–3.6)	60.0 (52–74)
Clone 22	0.23[a] (0.2–0.29)	2.0 (1.6–2.5)	26.0 (20–30)
Clone 2	0.12[a] (0.1–0.14)	0.9 (0.7–1.2)	7.5 (6.2–9.2)

[a] A second receptor population, with very low affinity, could be detected in these experiments. It was however not possible to determine its B_{max} accurately.

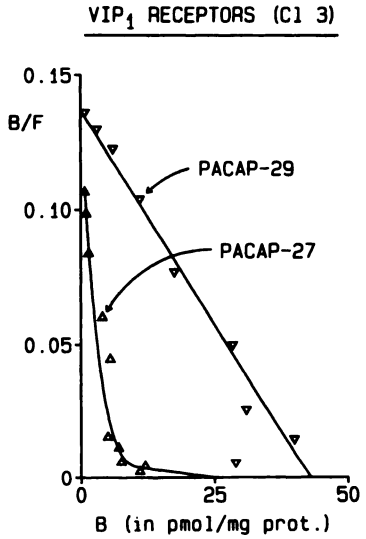

Fig. 3. Scatchard transformation of saturation curves obtained by incubating a tracer concentration of (△) ^{125}I-PACAP-27 or (▽) ^{125}I-PACAP-29 and increasing concentrations of the homologous unlabeled peptide with the use of membranes of CHO cells expressing VIP_1 receptors. *B*, expressed in pmol of tracer bound/mg of membrane protein. *B/F*, ratio of the bound tracer to the free tracer.

tracers in the other two N-PACAP/VIP_1 receptor clones with lower receptor densities (results not shown). The relative concentrations of receptors labeled with a high affinity by ^{125}I-VIP (super high sites) and by ^{125}I-PACAP-27 (super high and high sites) were constant, but the proportion of receptors found in the L state (difference between ^{125}I-PACAP-29 and ^{125}I-PACAP-27 B_{max}) increased with increasing receptor concentration (Table 3).

Discussion

Adenylate Cyclase Activation by Chimeric Receptors

The adenylate cyclase activation profile of PACAP I receptors and PACAP II/VIP_1 receptors is clearly different: PACAP I receptors are activated by low concentrations of PACAP-27 or PACAP-38 (EC_{50} = 2 nM), but very high concentrations of VIP are required to activate the receptors (EC_{50} = 2000 nM). In contrast, PACAP II/VIP_1 receptors are activated by the three peptides with the same EC_{50} value (2.5 nM). This reflects at least in part the different binding properties of the two receptors (Table 4).

The chimeric receptors were activated by lower concentrations of PACAP-27 compared with VIP, suggesting that the amino-terminal extracellular domain carries part of the discriminating domain of the receptor. A similar contribution of this receptor domain was also described for the secretin receptor (19, 20). The pharmacological properties of the chimeric receptor are nevertheless more like VIP_1 receptor than like PACAP I receptor. It should also be noted that in contrast to both natural receptors, the chimeric receptor had a higher affinity for PACAP-29 and PACAP-38 than for PACAP-27. This result indicated that the two (amino-terminal and core) domains influenced each other and that the

TABLE 4

Results of competition curves analysis.

Shown is the fraction of bound tracer labeling each affinity state (B_{SH}, B_H, and B_L) (in percent) and unlabeled peptide dissociation constants (K_{SH}, K_H, and K_L) (in nM) found by analysis of the competition curves obtained with the three tracers in CHO cell membranes expressing VIP_1 receptors, PACAP I receptors or N-PACAP/VIP_1 receptors.[a]

		^{125}I-VIP	^{125}I-PACAP-27	^{125}I-PACAP-29	
VIP_1 receptors					
VIP	SH		>80%, 0.5 nM	80%, 0.5 nM	20%, 0.5 nM
	H			20%, 30 nM	80%, 30 nM
PACAP-27	SH		>80%, 0.5 nM	80%, 0.5 nM	20%, 0.5 nM
	H			20%, 13 nM	80%, 13 nM
PACAP-38	SH		>80%, 1.5 nM	80%, 1.5 nM	20%, 1.5 nM
	H			20%, 5 nM	80%, 5.0 nM
PACAP I receptors					
VIP	SH		N.D.[a]	23%, 140 nM	
	H			77%, 3,000 nM	27%, 2,500 nM
	L				73%, >10,000 nM
PACAP-27	SH		N.D.	100%, 2.5 nM	27%, 2.5 nM
	H				73%, 25 nM
	L				
PACAP-38	SH		N.D.	100%, 2.0 nM	27%, 2 nM
	H				73%, 5 nM
	L				
N-PACAP/VIP_1 receptors					
VIP	SH		70%, 1 nM	20%, 1 nM	
	H		30%, ~30 nM[b]	65%, 30 nM	15%, ~30 nM
	L			15%, 2,000 nM	85%, 2,000 nM
PACAP-27	SH		70%, 0.7 nM	20%, 0.7 nM	
	H		30%, ~1.2 nM	65%, 1.2 nM	15%, ~1.2 nM
	L			15%, 50 nM	85%, 50 nM
PACAP-38	SH		70%, 0.9 nM	20%, 0.9 nM	
	H		30%, ~1.8 nM	65%, 1.8 nM	15%, ~1.8 nM
	L			15%, 3.0 nM	85%, 3 nM

[a] The proportions of SH/H/L states were determined with a 95% confidence interval of ±5–10%. The 95% confidence interval of the IC_{50} values was generally ±0.1–0.2 log unit (±20–30% of the average value).

[b] The IC_{50} values of the low affinity state labeled by ^{125}I-VIP and of the high affinity state labeled by ^{125}I-PACAP-29 in N-PACAP/VIP_1 receptors could not be determined with precision: this is indicated by the ~ sign.

N.D., not detectable.

conformation of either or both domains was slightly different in the chimeric receptor compared with the corresponding wild-type receptor.

Binding Studies

The only antagonists known to recognize PACAP I and PACAP II receptors have a weak (micromolar) potency; it is therefore not possible to perform binding studies with radiolabeled antagonists. Because agonists usually recognize in a different manner the receptor and the receptor/G protein complex, we decided to compare the binding properties of several radiolabeled agonists with the receptors discussed in the introductory paragraph.

Determination of the Receptor Densities

We were in general unable to achieve receptor saturation with the radioiodinated ligands in the concentration range amenable to study (<1.0 nM ^{125}I-peptide). We therefore assumed that radioiodination did not modify the peptides affinities, analyzed detailed homologous competition curves (^{125}I-peptide/peptide) as saturation curves (18), and compared the super high, high, and low B_{max} values obtained with each tracer.

^{125}I-VIP and unlabeled VIP have the same affinity for recombinant VIP_1 receptors in CHO cells (current study and Ref. 10). The same is true for ^{125}I-PACAP-27 and ^{125}I-PACAP-38 binding to the VIP_1 receptors in rat liver (17).

The relative B_{max} value for ^{125}I-VIP compared with ^{125}I-PACAP-29 (5 compared with 45 pmol/mg of protein) was in good agreement with the proportion of sites with high/low affinity for VIP labeled by ^{125}I-PACAP-29 (20 ± 5%). This suggested that radioiodination did not modify the affinity of PACAP-29 for recombinant VIP_1 receptors. The same line of argument could be applied to ^{125}I-PACAP-27.

Unfortunately, we were unable to perform ^{125}I-peptide saturation curves on CHO cell membranes expressing the normal PACAP-I receptor or the N-PACAP/VIP_1 chimeric receptors. This reflects the rather low affinities of the peptides for these receptors; the unlabeled PACAP-27 and PACAP-38 binding affinities for recombinant PACAP-I receptors were ≤10-fold lower than the affinities observed in brain tissues or cell lines (12, 13). This discrepancy might reflect the existence of as many as five PACAP I receptor isoforms (due to alternative splicing) in brain and cell lines (9), only one of which was expressed in the CHO cell line used in the current study. We indeed previously observed that the HOP-PACAP I receptor (expressed in CHO cells), had a slightly greater affinity than the normal PACAP I receptor for PACAP-27 (10). Further experiments are, however, necessary to confirm this hypothesis.

The relative ^{125}I-VIP/^{125}I-PACAP-27/^{125}I-PACAP-29 *B*max values and the proportions of super high, high, and low sites labeled by each radioligand were in good agreement, suggesting that radioiodination did not affect one peptide more than the other. Because the three radioligands had the same affinity as the unlabeled ligands for VIP_1 receptors (see above), as well as for rat brain and spinal cord and for AR 4–2J cells PACAP I receptors (unidentified isoforms) (12, 13,

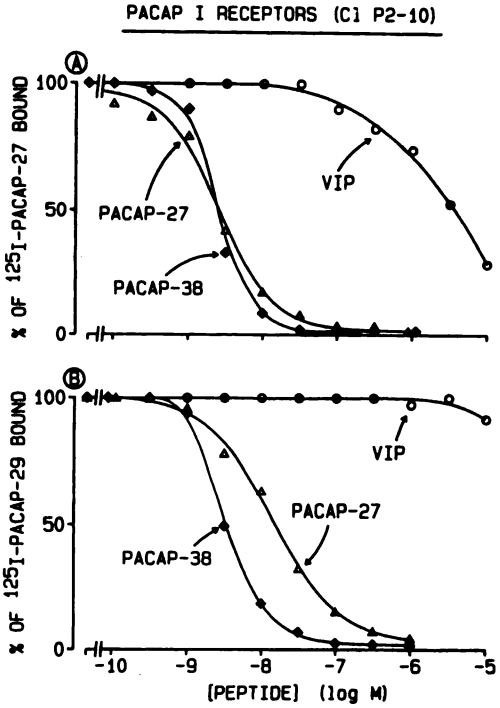

Fig. 4. Inhibition of (A) ^{125}I-PACAP-27 and (B) ^{125}I-PACAP-29 binding to membranes of CHO cells expressing PACAP I receptors (clone P2-10) by increasing concentrations of (△) PACAP-27, (♦) PACAP-38, and (○) VIP. The results were expressed in percentage of tracer bound in absence of added peptides and are the average of three to seven experiments performed in duplicate.

15), it is likely that they also had the same affinity for the normal PACAP I receptors and for the chimeric receptor.

Identity of the PACAP A and B Receptor Subtypes

^{125}I-PACAP-29 labeled at least two receptor populations in CHO cells expressing PACAP I receptors; 27% of the ^{125}I-PACAP-29-labeled receptors (super high and high receptors) were also labeled by ^{125}I-PACAP-27 and did not discriminate between the two natural PACAP forms (PACAP-27 and PACAP-38). In contrast, the majority of the ^{125}I-PACAP-29-labeled receptors (low receptors) recognized preferentially PACAP-38 over PACAP-27 and could not be labeled by ^{125}I-PACAP-27. The CHO cells used in this study had been transfected with a single plasmid, encoding the normal (shortest) form of the PACAP I receptor. This result therefore suggested that the previously described A and B PACAP receptor subtypes (12, 13) in fact correspond to different states (super high plus high and low) of the same protein. The addition of 10 μM GTP to the incubation medium markedly inhibited ^{125}I-PACAP-27 binding (see Fig. 2). In contrast, ^{125}I-PACAP-29/PACAP-29 and PACAP-38 competition curves (and the ^{125}I-PACAP-29 binding site density) were not affected by the addition of GTP to the concentration medium (data not shown). This result suggested that PACAP-27 recognized with a higher affinity the receptor/G protein complex (A receptors), whereas ^{125}I-PACAP-29 recognized indifferently both receptor populations: the receptor/G protein complex (A receptors) as well as uncoupled receptors in excess over the G protein (B receptors).

Labeling of the PACAP II/VIP$_1$ receptors. ^{125}I-VIP labeled sites with a high affinity for VIP (IC_{50} = 0.5 nM).

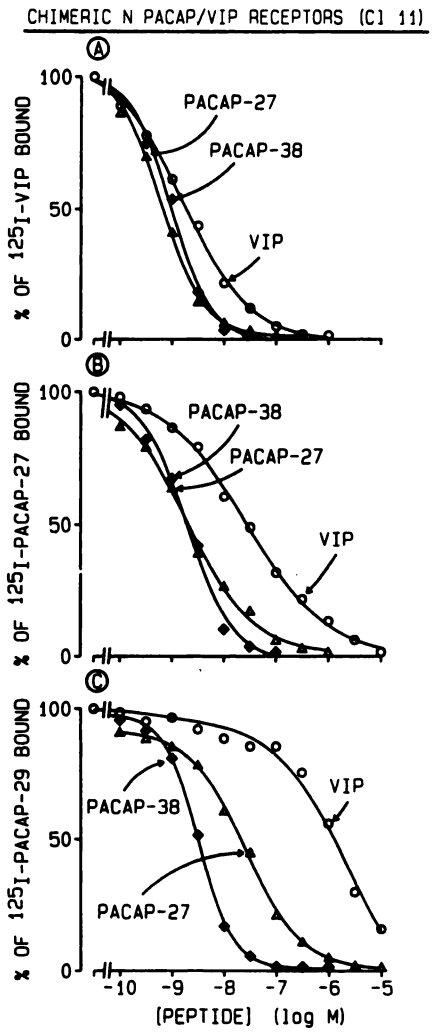

Fig. 5. Inhibition of (A) ^{125}I-VIP, (B) ^{125}I-PACAP-27, and (C) ^{125}I-PACAP-29 binding to membranes of CHO cells expressing chimeric N-PACAP/VIP$_1$ receptors (clone 11) through increasing the concentrations of (△) PACAP-27, (♦) PACAP-38, and (○) VIP. The results were expressed in percentage of tracer bound in absence of added peptides and are the average of three to seven experiments performed in duplicate.

^{125}I-PACAP-27 labeled the same receptor population but also detected the existence of a second receptor with low affinity for VIP and PACAP-27. ^{125}I-PACAP-29 labeled nonselectively the two receptor populations.

^{125}I-VIP and ^{125}I-PACAP-27 bindings were significantly inhibited by the addition of GTP to the incubation medium (Fig. 2). In contrast, ^{125}I-PACAP-29 binding was only slightly inhibited by GTP, if at all, and the receptor density labeled by this tracer was not affected by the addition of 10 μM GTP to the medium (data results not shown).

As for the PACAP I receptor, the results can be explained by assuming that ^{125}I-VIP, ^{125}I-PACAP-27, and ^{125}I-PACAP-29 differ in their selectivity for the receptor/G protein complex as opposed to the uncoupled receptor. The results suggested that ^{125}I-VIP and ^{125}I-PACAP-27 labeled almost exclusively the ternary complex and that ^{125}I-PACAP-29 recognized both receptor states with the same affinity.

Labeling of the chimeric N-PACAP/VIP$_1$ receptors. The tracers ^{125}I-VIP, ^{125}I-PACAP-27, and ^{125}I-PACAP-29

were able to label the chimeric N-PACAP/VIP_1 receptors expressed in CHO cells. Like the two parent receptors, ^{125}I-PACAP-27 labeled preferentially a GTP-sensitive subpopulation of the receptors, whereas ^{125}I-PACAP-29 also labeled a much larger, GTP-insensitive receptor population, and the ^{125}I-PACAP-29 receptor density was not affected by GTP (10 μM) (not shown). This result suggested that ^{125}I-PACAP-27 labeled only receptor/G protein complexes, whereas ^{125}I-PACAP-29 labeled the total (coupled plus uncoupled) receptor population.

Comparison with the classic ternary complex model. The classic ternary complex model (21) predicts that the G protein-coupled receptors can be found in two states: G protein-bound receptors with a high affinity for agonists and uncoupled receptors with a low affinity for agonists. This model does not predict three agonist binding states in proportions comparable to the VIP binding states observed in this current study for PACAP I or chimeric N-PACAP/VIP_1 receptors (22). We suggest three alternative explanations for this phenomenon: (i) G protein-coupled receptors are usually able to recognize preferentially one particular set of G proteins but may possess a weaker but significant affinity for a second G protein family. Because the apparent affinity of the ternary complex for the agonist depends in part on the affinity of the receptor/agonist complex for the G protein, it is possible that the super high and high receptors (with super high and high affinity for VIP) correspond to PACAP-I or chimeric N-PACAP-VIP_1 receptors associated with different G proteins. It is indeed known that PACAP I receptors are able to stimulate not only adenylate cyclase (via G_S) but also phospholipase C (probably through G proteins of the $G_{q/11}$ family) (9, 23–25). (ii) When agonists bind to the receptors, they favor the dissociation of GDP from the G proteins. If this GDP is trapped in an unstirred layer close to the ternary complex, its concentration will increase with increasing agonist binding, and this will result in a progressive decrease of the average agonist affinity for the remaining receptors. Because VIP seems to discriminate the coupled and uncoupled receptors much more strongly than PACAP, it might seem to recognize three receptor states in some membranes. (iii) It is usually assumed that like GTP, GDP prevents a stable interaction between the agonist/receptor complex and the G protein/GDP complex. If VIP had a very strong (favorable) effect on the G protein/GDP complex recognition by the receptors but a weaker effect on GDP release, it is possible that VIP discriminated three receptor states: VIP/receptor/G protein empty, VIP/receptor/G protein/GDP, and (uncoupled) VIP/receptor complexes.

The very large majority of agonists discriminate two receptor states in the absence of GTP: coupled (high affinity) receptors and uncoupled (low affinity) receptors. It is therefore assumed that facilitated receptor/G protein recognition is essential to allow G protein activation. PACAP-29, which does not seem to discriminate these two receptor states, nevertheless behaves as a full agonist in functional studies (Fig. 1). To explain this observation, we should like to point out that receptor recognition per mean ± standard error is not sufficient for G protein activation: GTP binding is also necessary. Agonist and guanyl-nucleotide recognition (by the receptor and G protein), is mutually exclusive; agonists activate G proteins by facilitating GDP release. If the PACAP-29/receptor complex accelerates the guanyl-nucleotide association and dissociation to the same extent, it will not change the affinity of GTP for the G protein at equilibrium (and therefore GTP will not affect PACAP-29 binding) but will facilitate GTP binding to the G protein (and therefore activate G_S).

Comparison of binding and adenylate cyclase activation in the determination of receptor density. Most of the receptors labeled by ^{125}I-PACAP-29 had a slightly different binding profile than the ^{125}I-PACAP-27-labeled sites. It was therefore important to determine whether the ^{125}I-PACAP-29 receptors were functional (as opposed to misfolded) receptors. To answer this question, we took advantage of the fact that the chimeric N-PACAP/VIP_1 receptors had apparently been expressed to an unusually high level in clone 11, and we tested several other clones to find cells with a lower receptor density. The results obtained in two other clones (clones 2 and 22) with 2.4- and 8-fold, respectively, less ^{125}I-PACAP-29 binding sites are summarized (Tables 2 and 3). As shown in Table 3, the receptor density labeled by ^{125}I-VIP and ^{125}I-PACAP-27 was ~2-fold greater in clone 22 than in 2 and not significantly different in clones 11 and 22. In contrast, we observed a significant difference between the EC_{50} values of each peptide in the three clones (Table 1); the EC_{50} values were inversely proportional to the density of ^{125}I-PACAP-29 receptors, suggesting that this peptide was capable of correctly labeling all of the spare receptors in the three cell lines.

We were unable to use the same approach with the clones expressing PACAP I receptors and PACAP II/VIP_1 receptors because the relative densities of ^{125}I-VIP-, ^{125}I-PACAP-27-, and ^{125}I-PACAP-29-labeled receptors were constant. The results of Spengler *et al.* (9), who suggested that PACAP-27 and PACAP-38 activate the phospholipase C in transfected LLC-PK9 cells expressing PACAP I receptors with very high EC_{50} values and that PACAP-38 is more potent than PACAP-27, support the hypothesis that the PACAP-38-preferring, low affinity low receptor state labeled by ^{125}I-PACAP-29 is indeed functional.

Identity of the active state. The binding profile of the ^{125}I-PACAP-29-labeled receptors is different from the binding profile of the ^{125}I-PACAP-27-labeled receptors. It is therefore very tempting to attempt to identify the active state of the receptors through a comparison of the VIP-, PACAP-27-, and PACAP-38-binding and adenylate cyclase or phospholipase C activation profiles. We, however, think that this search is futile, with both receptor states being necessary for G protein activation. Indeed, if the agonist/receptor complex did not have a high affinity for the empty G protein, it would be unable to promote GDP dissociation from the α subunit and G protein activation. Formation of the high affinity ternary complex (which accumulates in the absence of GTP in binding studies) is therefore essential for receptor signaling, and the ternary complex is an active state of the receptor. If, however, the agonist/receptor complex was unable to dissociate from the G protein/GTP complex, the receptor would be unable to catalyze the activation of other G proteins. The low affinity (uncoupled) agonist/receptor complex that accumulates in the presence of GTP is therefore also an active state of the receptors. This might explain why the adenylate cyclase stimulation profile observed with the three receptors studied in this study was intermediate between the high affinity and low affinity receptor binding profiles.

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