Maxadilan, the Vasodilator from Sand Flies, Is a Specific Pituitary Adenylate Cyclase Activating Peptide Type I Receptor Agonist

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Maxadilan is a potent vasodilator peptide isolated from salivary gland lysates of the sand fly Lutzomyia longipalpis, a vector of leishmaniasis. The peptide aids the fly in obtaining blood from the skin of its vertebrate hosts but the mammalian receptor through which this insect ligand acts was unknown. We demonstrate that maxadilan is an agonist of the type I receptor for pituitary adenylate cyclase activating peptide, a neuropeptide with vascular activity. This surprising observation is a unique example of convergent evolution from a functional standpoint as these two peptides do not share significant sequence homology.

Hematophagous arthropods have developed a variety of anti-hemostatic compounds to ensure success in obtaining blood from their hosts (1). The compounds described to date tend to act via inhibition of biochemical pathways. As examples, certain ticks block coagulation via an inhibitor of factor Xa while mosquitoes and deerflies contain apprasyne and a glycoprotein IIb/IIIa antagonist, respectively, that block platelet aggregation (2–4). In contrast, salivary gland lysates of the bloodfeeding sand fly Lutzomyia longipalpis contain maxadilan, a potent and persistent vasodilator of human skin that might be expected to function as an agonist at its cognate vertebrate receptor (5–7). As flies are evolutionary distant from vertebrates and maxadilan does not share sequence homology with other known peptides, we postulated that this peptide would act either at an orphan receptor whose endogenous ligand was unknown or at a receptor whose ligand had a structure distinct from maxadilan.

Maxadilan is synthesized as a prepropeptide that undergoes signal peptide and C-terminal cleavage and associated amidation to a 61-amino acid peptide. It is an endothelium-independent vasodilator with immunomodulatory properties that binds to a cell surface receptor, inducing the accumulation of intracellular cAMP and subsequent vasodilation (8, 9). Examination of the distribution of receptor sites revealed that the peptide binds to brain extracts and neural crest-derived cell lines (10, 11). Initial studies focused on calcitonin gene-related peptide, a neuropeptide with vasodilatory and immunomodulatory properties that, upon injection into skin, produces a pattern of erythema identical to that induced by maxadilan (12, 13). Despite these similarities, it was found that maxadilan does not bind to calcitonin gene-related peptide receptors. The effect of maxadilan on vascular and neural tissues prompted an examination of the capacity for maxadilan to compete with the binding of other neuropeptides, that also have vascular actions, to their cognate receptors and to functionally activate these receptors.

MATERIALS AND METHODS

Preparation of Peptides—Recombinant maxadilan produced in Escherichia coli contained the four additional amino acid residues glycine, serine, isoleucine, and leucine at the N terminus as a result of construction in the pGEX vector designed for cleavage with thrombin. Termed “GSIL-maxadilan,” it was purified to homogeneity using reverse phase high performance liquid chromatography (10). 125I-Maxadilan with a specific activity of 2000 Ci/nmol was prepared using the chloramine-T method (10). 125I-PACAP‡ was purchased from DuPont NEN and 125I-VIP was purchased from Amersham. PACAP 27, PACAP 38, and VIP were obtained from Peninsula Laboratories (Belmont, CA).

COS Cell Transfection—Plasmids preparations encoding the three PACAP receptor cDNAs were prepared and purified twice over cesium chloride gradients. COS cells were plated in 6-well plates (3 × 10⁵ cells/well for binding assays) or 12-well plates (1.5 × 10⁶ cells/well for assay of cAMP) the previous day. On the day of transfection, medium was aspirated and cells were rinsed gently twice with 1 ml of 37°C phosphate-buffered saline without Ca²⁺ or Mg²⁺. DNA solution (475 μl of phosphate-buffered saline without Ca²⁺ or Mg²⁺, 1 μg of DNA, and 25 μl of 10 mg/ml DEA鑫-dextran solution) was added to the well. Cells were incubated 37°C for 30 min with occasional swirling. 3 ml of media (10% Nuserm, penicillin/streptomycin, glutamine in Dulbecco’s modified Eagle’s medium) containing 80 μg/ml chloroquine was added and cells were incubated for 3 h at 37°C. DNA/DEA鑫-dextran/chloroquine solution was aspirated and the cells were treated with 1 ml of 10% dimethyl sulfoxide made with complete media for 2.5 min. Fresh medium was added, and the cells were assayed 3 days later. The sources of the receptor clones were rat for PACAP type I and human for VIP types I and II.

Preparation of Tissue Membranes—Rat and rabbit brain tissue were obtained from Pel-Freez (Rogers, AR). Membrane fractions from tissues were prepared as described previously (6). Briefly, tissue was placed in 10 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.6) containing 0.32 M sucrose, 5 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 μg/ml bacitracin, and 10 μg/ml phenylmethylsulfonyl fluoride. Tissue was homogenized with a Polytron PT 3000 (Brinkmann Instruments, Westbury, NY) for 30 s at power level 8 at 4°C and the homogenate was centrifuged for 10 min at 1,000 × g at 4°C. The supernatant was removed, and the pellet resuspended in 15 ml of homogenizing buffer, homogenized again using the Polytron at the same setting as the first homogenization, and centrifuged at 1,000 × g for 10 min at 4°C. The combined supernatant was centrifuged at 30,000 × g for 20 min at 4°C. The pellet was washed twice by successive suspension in 50 mM Tris-HCl buffer containing 1 mM MgCl₂, 0.3% bovine serum albumin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 μg/ml bacitracin, and 10 μg/ml phenylmethylsulfonyl fluoride.

Binding Study—Crude membranes (250–400 μg) were incubated for 2 h at 4°C in a final volume of 0.5 ml consisting of 50 mM Tris-HCl buffer (pH 7.6) containing 0.3% bovine serum albumin, 1 mM MgCl₂, 1

1 The abbreviations used are: PACAP, pituitary adenylate cyclase activating peptide; VIP, vasoactive intestinal peptide.

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μg/ml leupeptin, 1 μg/ml pepstatin A, 2 μg/ml bacitracin, 10 μg/ml phenylmethylsulfonyl fluoride, and 50 μM 125I-maxadilan or 125I-PACAP 27 in the absence or presence of 1 μM maxadilan or PACAP 38. For the binding assay using transfected COS cell, cells were incubated with 50 μM labeled peptide in the presence of 1 μM of the indicated competitor ligand (4). At the end of incubation, samples were assayed for protein bound radioactivity by vacuum filtration through GF/C Whatman glass microfiber filters pretreated with 0.5% polyethyleneimine. Filters were washed three times with 3 ml of buffer consisting of 50 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, and 0.3% bovine serum albumin at 4°C. The radioactivity trapped on the filters was measured using a γ-counter. Nonspecific binding represented between 10 and 20% of total binding. Percent of specific binding in the presence of competitor is shown. Percent of specific binding for the control was defined to be 100% as follows: (total binding of 50 μM 125I-maxadilan – binding of 125I-maxadilan in the presence of 1 μM cold maxadilan)/(total binding of 125I-maxadilan) × 100. Percent of specific binding for the peptides is defined as (binding of 125I-maxadilan in the presence of 1 μM cold competitor)/(total binding of 125I-maxadilan – binding of 125I-maxadilan in the presence of 1 μM cold maxadilan) × 100. Proteins were estimated by the method of Bradford using bovine serum albumin as standard. All experiments were performed in triplicate on at least three occasions and the data displayed with standard deviations.

Measurement of Cyclic AMP—Transfected COS cells grown to confluence in 12-well plates were stimulated for 10 min with the indicated concentrations of ligand in Hank’s medium with 1 mM 3-isobutyl-1-methylxanthine and 0.1% bovine serum albumin. The incubation was terminated by aspiration of medium and addition of 400 μl of ice-cold 50 mM Tris, 4 mM EDTA (pH 7.5). The cells were harvested, transferred to 1.5-ml Eppendorf tubes, boiled for 5 min, and spun at top speed in an Eppendorf centrifuge for 5 min. Supernatants were collected and 50-μl aliquots were assayed using a kit for determination of cyclic AMP following the instructions of the manufacturer (Amersham). NBfl cells grown to confluence in 24-well plates were stimulated for 10 min with the 1 nM maxadilan or PACAP 38 in increasing concentrations PACAP (6–38) in Hank’s medium with 1 mM 3-isobutyl-1-methylxanthine and 0.1% bovine serum albumin. All experiments were performed in triplicate on at least three occasions and the data displayed with standard deviations.

RESULTS

Competition between 125I-Maxadilan and Peptides of the PACAP/VIP/Secretin Family—The ability of the peptides of the PACAP/VIP/secretin family to compete with labeled maxadilan in rat whole brain homogenates was determined by competition. Unlabeled maxadilan, PACAP 27, and PACAP 38 were able to compete with the binding of 125I-maxadilan to this preparation, in contrast to VIP, growth hormone releasing factor, glucagon, secretin, or peptide histidine isoleucine which did not compete substantially (Fig. 1).

Binding of Maxadilan to PACAP Receptor Subtypes—Maxadilan and PACAP 38 but not VIP competed with 125I-maxadilan for binding, consistent with 125I-maxadilan binding specifically to the PACAP type I receptor. Cells expressing VIP...
receptors types I and II (the remaining bars) were incubated with \(^{125}\text{I}\)-VIP in the presence of 1 \(\mu\text{M}\) unlabeled maxadilan, PACAP 38, or VIP. PACAP 38 and VIP but not maxadilan competed with \(^{125}\text{I}\)-VIP for binding, indicating that maxadilan does not compete with \(^{125}\text{I}\)-VIP for binding to the VIP types I and II receptors (Fig. 2A). Cells expressing PACAP type I, VIP type I, and VIP type II receptors were incubated with \(^{125}\text{I}\)-PACAP in the presence of unlabeled maxadilan, PACAP 38, or VIP. Maxadilan and PACAP 38 but not VIP competed with \(^{125}\text{I}\)-PACAP for binding to the PACAP type I receptor expressing cells. PACAP 38 and VIP but not maxadilan competed with \(^{125}\text{I}\)-PACAP for binding to cells expressing VIP type I and II receptors, indicating that maxadilan does not compete with \(^{125}\text{I}\)-PACAP for binding to these receptors (Fig. 2B).

**Concentration-dependent Competitive Binding Assay**—\(^{125}\text{I}\)-Maxadilan and \(^{125}\text{I}\)-PACAP binding to rat brain homogenates was concentration-dependently competed with unlabeled maxadilan, PACAP27, and PACPA38 but not VIP (Fig. 3, A and B). This result is consistent with maxadilan and PACAP sharing binding properties to rat brain membrane homogenates with similar affinities (Table 1).

**Accumulation of cAMP in COS Cells Transfected with PACAP/VIP Receptors: Induced by Maxadilan**—Maxadilan induced the accumulation of cAMP only in PACAP type I receptor expressing cells. In contrast, PACAP 38 induced the accumulation of cAMP in cells expressing each receptor while VIP was most effective at inducing the accumulation of cAMP in cells expressing VIP type I and II receptors (Fig. 4A). Although the

**TABLE I**

Relative affinities of the PACAP receptor in rat brain crude membrane for various peptides

| Peptide      | \(^{125}\text{I}\)-Maxadilan | \(^{125}\text{I}\)-PACAP 27 |
|--------------|------------------------------|--------------------------|
| Maxadilan    | 1.14 ± 0.07                 | 3.35 ± 0.29              |
| PACAP 38     | 1.91 ± 0.13                 | 4.45 ± 0.22              |
| PACAP 27     | 2.16 ± 0.45                 | 9.55 ± 0.75              |
| PACAP 6–38   | 8.4 ± 2.0                   | 39.1 ± 3.0               |
| VIP          | >1000                        | >1000                    |

**Fig. 3.** \(^{125}\text{I}\)-Maxadilan and \(^{125}\text{I}\)-PACAP bind to membrane homogenates from rat brain. Concentration-dependent competition of \(^{125}\text{I}\)-maxadilan (A) and \(^{125}\text{I}\)-PACAP 27 (B) binding to membrane homogenates from rat brain by maxadilan, PACAP 27, PACAP 38, and VIP.

**Fig. 4.** Accumulation of cAMP in COS cells transfected with PACAP/VIP receptors. A, accumulation of cAMP was measured by stimulation of maxadilan, PACAP 38, and VIP as described under “Materials and Methods.” 1, control; 2, maxadilan; 3, PACAP 27; 4, VIP. B, accumulation of cAMP in COS cells transfected with PACAP type I receptors. The EC\(_{50}\) for maxadilan was 0.62 ± 0.18 nM and that for PACAP 38 was 0.34 ± 0.08 nM. Data is expressed as cAMP accumulation per 10\(^6\) cells.
affinity of VIP for the PACAP type I receptor is 1000-fold less than PACAP, the presence of high numbers of receptors in the transfected cells combined with the amplification cascade of second messenger generation results in the generation of significant quantities of cAMP (Fig. 4A). Both maxadilan and PACAP 38 induced the accumulation of cAMP in COS cells transfected with PACAP type I receptors in a concentration-dependent fashion (Fig. 4B).

Inhibition of cAMP Accumulation in NBfl Cells—Maxadilan and PACAP induced accumulation of cAMP in NBfl cells was inhibited by the antagonist PACAP (6–38) in a concentration-dependent fashion and to the same degree (Fig. 5).

DISCUSSION

Examination of several neural crest derived cell lines revealed that maxadilan bound to the rat pheochromocytoma line PC12 and the human neuroblastoma line NBfl, features shared by ligands of the PACAP/VIP/secretin/glucagon family (14, 15) leading to an evaluation of members of this receptor family for their capacity to interact with maxadilan. The binding of 125I-maxadilan to crude membrane homogenates of rat brain could be inhibited by unlabeled maxadilan, PACAP 27, and PACAP 38 but not substantially by VIP, growth hormone releasing factor (GRF), glucagon, secretin, or peptide histidine isoleucine (Fig. 1).

PACAP was first identified from ovine hypothalamus because of its potent activity in stimulating cAMP production in rat anterior pituitary cells (16). Since its isolation, a wealth of information has been accumulated describing its activity in a variety of areas including brain, adrenal and vascular tissues, endocrine and immune responses. It has been postulated that this peptide has important functions ranging from the control of hypothalamic-pituitary communication to insulin secretion while PACAP-like neurotransmission has been demonstrated in Drosophila (17, 18). Two functional forms of PACAP have been found, a short form, designated PACAP 27 and a long form, designated PACAP 38. Cloning of the PACAP receptors reveals three distinct subtypes and at least four variants produced by alternative splicing (19–22). Two of the three receptors also function as receptors for VIP and pose a nomenclature issue. Here, they are referred to as PACAP type I, VIP type I (PACAP type II), and VIP type II (PACAP type III). The PACAP type I receptor is not considered a receptor for VIP as its affinity for this ligand is 10^3 times less than its affinity for PACAP.

To examine in more detail the interaction of maxadilan with PACAP/VIP receptors, the three known PACAP receptor subtypes were expressed transiently in COS cells. 125I-Maxadilan bound to cells expressing PACAP type I receptors and this binding could be inhibited by unlabeled maxadilan and PACAP but not VIP (Fig. 2A). The binding of 125I-VIP to cells expressing VIP types I and II receptors was not affected by maxadilan (Fig. 2A). Maxadilan competed with the binding of 125I-PACAP to cells expressing PACAP type I receptors but not those expressing VIP type I or VIP type II receptors (Fig. 2B). These results indicate that maxadilan binds specifically to the PACAP type I receptor and competes spatially with the binding of PACAP and vice versa. As PACAP type I receptors predominate in brain, the binding of 125I-maxadilan as compared to 125I-PACAP was examined in crude membrane homogenates of rat brain (Fig. 3, A and B). Similar competition curves were obtained for the two peptides leading to the conclusion that they have similar affinities for receptors in rat brain (Table I).

The accumulation of cAMP by maxadilan and PACAP was examined in COS cells transfected with the individual PACAP receptor clones. Maxadilan induced the accumulation of cAMP only in COS cells expressing PACAP type I receptors while PACAP induced significant levels of this second messenger in cells transfected with each receptor (Fig. 4A). The induction of cAMP by VIP in cells expressing type II and III receptors was similar to the response induced by PACAP. The effect of VIP on cells expressing type I receptors was markedly less than either PACAP or maxadilan (Fig. 4A). The effect of maxadilan was concentration-dependent in cells expressing type I receptors, overlapping that of PACAP (Fig. 4B). The PACAP antagonist (PACAP 6–38) inhibited in a concentration-dependent fashion, and to the same degree, the cAMP responses of maxadilan and PACAP 38 in NBfl human neuroblastoma cells which predominantly express PACAP type I receptors (Fig. 5).

There are examples in which a ligand from one species is homologous to a ligand from another and they each bind to the same receptor. Thus, bombesin, from amphibian skin, and gastrin releasing peptide, which have virtually identical N-terminal sequences, each act on the bombesin receptor, while sarafotoxin, from snake venom, is 66% identical to endothelin I, and each activates the endothelin receptor (23, 24). A similar scenario exists for helodermin, a lizard peptide, and VIP, which share more than 50% sequence homology (25). That maxadilan and PACAP compete with each other for activation of the same receptor is interesting in that the peptides do not share significant primary sequence homology (Fig. 6). Structural studies on PACAP suggest that residues 2–4 and 8–11 form a b-sheet between the two helices (11). The overall predicted structural similarities between maxadilan and PACAP may account for the virtually identical binding noted for these two peptides. Although mutational analysis of the VIP and

![Fig. 5. Effect of PACAP antagonist PACAP (6–38) on accumulation of cAMP.](image)

**FIG. 6. Sequence alignment of maxadilan and the PACAP/VIP family of peptides.** The complete amino acid sequences of the peptides are presented. The sequences for maxadilan and the PACAP/VIP family of peptides have been arbitrarily arranged such that the shared phenylalanine/threonine pair and common C-terminal lysines and valine are aligned.
PACAP ligands are shedding some light on the relative importance of specific amino acid residues in these peptides vis-à-vis receptor specificity, their applicability to an explanation for the type I receptor-specific affinity of maxadilan is lacking.

In conclusion, maxadilan, a peptide derived from sand flies, does not share sequence homology with PACAP, an endogenous mammalian peptide, yet binds to the same site as PACAP and functionally activates the PACAP type I receptor. The use of maxadilan as a PACAP type I receptor-specific agonist, and generation of antagonists based on the sequence of maxadilan, should be helpful in defining further the function of this receptor and its importance in vascular, endocrine, and neurological phenomena.

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