Maxadilan, a 61-amino-acid vasodilatory peptide, was initially isolated from the salivary glands of the sand fly Lutzomyia longipalpis. Although its primary sequence has no homology to that of pituitary adenylyl cyclase-activating peptide, maxadilan is an agonist for the PAC1 receptor. A total of 58 substitution and deletion mutants was engineered in an effort to determine which residues were important for receptor activation. The mutants were characterized functionally using an assay based on pigment granule translocation in PAC1-expressing Xenopus laevis melanophores. Substitution of charged residues and proline 43 could alter (but not eliminate) the agonist activity of the mutants. In contrast, we found that several multiple substitution mutants of the predicted β-strand threonine residues became antagonists at the PAC1 receptor. The results suggest that these threonine residues are cooperatively involved in PAC1 activation.

Maxadilan specifically activates PAC1, the type I receptor for pituitary adenylyl cyclase-activating peptide (PACAP). Maxadilan does not activate the other two receptors for PACAP, VPAC1, and VPAC2. PAC1 is a seven-transmembrane-domain G protein-coupled receptor first cloned from a pancreatic acinar carcinoma cell line. It is expressed in the nervous system and in a wide variety of peripheral tissues. Surprisingly, maxadilan and PACAP do not share significant sequence homology. The functional activation of the receptor requires the C-terminal 1–5 and 14–51. The ring from 1–5 is not needed for agonist activity, but alterations of the cysteines at 14 or 51 eliminate activity. This larger ring is presumably needed for the structural integrity of the peptide and possibly the interaction with the PAC1 receptor. Of note, deletion of the amino acids 25–41 of the β-strand generates a specific PAC1 receptor antagonist, M65, suggesting that this region is important for functional activation of the receptor.

The importance of particular residues in receptor activation might be ascertained by substitution mutations in the absence of crystal or NMR structures for maxadilan. Some of this mutational analysis has been provided naturally, as sequence alterations in 21 of the 61 amino acid residues of maxadilan have been reported among field populations as well as among sibling species of sand flies. Maxadilan variants differed by as much as 23% in amino acid composition. All of these natural variants shared the same degree of vasodilatory activity and had similar predicted secondary structures and hydrophobicity plots. It has been suggested that this polymorphism is a form of antigenic variation to escape host immune responses. A subsequent study confirmed antigenic specificity to the C-terminal end of variant maxadilan peptides.

Because charged residues are often important to function, single or multiple point substitutions to the alanine of selected charged residues throughout the maxadilan molecule were generated. In addition to the polar residues, proline 43 was mutated to alanine, because proline residues can form turns in the polypeptide chain, with potential functional implications. Furthermore, as described above, the 25–41-amino-acid segment, deleted in M65, may be important in the functional activation of PAC1. This region contains six threonine residues, which are potentially involved in the formation of hydrogen bonds with PAC1 residues. To investigate this hypothesis, we engineered substitution mutations of these threonine residues to alanine. Three deletion mutants were also designed to further evaluate amino acid regions involved in peptide-receptor interactions.

To evaluate the interaction of these mutants with the PAC1 receptor, we used a functional assay based on pigment granule translocation in Xenopus laevis melanophores stably expressing PAC1 (10). Activation of PAC1 in melanophores leads to an increase in intracellular levels of cAMP with subsequent melanosome dispersion. This phenomenon is readily quantified via changes in phototransmission (10).

**MATERIALS AND METHODS**

**Melanophore Cell Culture**—Frog fibroblasts were cultured at room temperature for 3–4 days in 0.7× Lebowitz-15 medium, 20% fetal bovine serum, 1× glutamine, and 1× penicillin-streptomycin. This conditioned medium was used to maintain a stable line of melanophores expressing the rat PAC1 receptor (10).

**Maxadilan Expression Vector**—The cDNA (210 bp) of maxadilan (11) was cloned as an Ncol-XhoI fragment into the pTWIN1 vector (New England Biolabs, Beverly, MA) with its N terminus fused to the chitin binding domain and a mini-intein from the Synechocystis sp. dnaB vector.
Mutational Analysis of Maxadilan

FIGURE 1. Alignment of amino acids of maxadilan, PACAP38, PACAP27, VIP, secretin, and glucagon showing lack of any significant homology. The alignment is arbitrary, allowing the phenylalanine at position 34 in maxadilan to match the identical residue in the other peptides. Bold letters indicate maxadilan residues mutated to alanine in this study. Underlined letters indicate naturally variable residues (9). Italics indicate the 25–41-amino-acid segment deleted in the specific PAC1 antagonist M65 (8). The single letter notation for amino acids is used.

TABLE 1
The potency of maxadilan single mutants affecting charged residues and proline 43

| Maxadilan mutants | EC50 maxadilan/EC50 mutant |
|-------------------|-----------------------------|
| N45A              | 2.6                         |
| K49A              | 2                           |
| P43A              | 2                           |
| D38A              | 2                           |
| D12A              | 1                           |
| D13A              | 1                           |
| E50A              | 1                           |
| K53A              | 1                           |
| K55A              | 1                           |
| K56A              | 0.8                         |
| K57A              | 0.8                         |
| E58A              | 0.8                         |
| K60A              | 0.8                         |
| N22A              | 0.5                         |
| R8A               | 0.1                         |
| K9A               | 0.1                         |
| K16A              | 0.1                         |
| H19A              | 0.1                         |
| H20A              | 0.1                         |

TABLE 2
The potency of maxadilan multiple mutants affecting charged residues and proline 43

| Maxadilan mutants | EC50 maxadilan/EC50 mutant |
|-------------------|-----------------------------|
| R8A/K9A/D12A/D13A/H19A/H20A/N22A | 0.5                 |
| R8A/K9A/D12A/D13A/K16A/H19A/H20A/N22A | 0.33                |
| D38A/N45A         | 0.2                         |
| R8A/D38A/P43A/N45A | 0.2                 |
| K53A/K57A         | 0.16                        |
| H19A/H20A/N22A    | 0.16                        |
| D38A/P43A         | 0.13                        |
| K9A/D38A/P43A/N45A | 0.11                |
| K16A/D38A/P43A/N45A | 0.11                |
| K53A/K55A/K56A    | 0.1                         |
| K55A/K56A/K57A    | 0.08                        |
| K53A/K55A/K57A    | 0.07                        |
| D38A/P43A/N45A    | 0.06                        |
| K53A/K55A/K56A/K57A | 0.008              |

TABLE 3
The potencies of the maxadilan threonine → alanine mutants that maintained agonist activity at the PAC1 receptor

| Maxadilan mutants | EC50 maxadilan/EC50 mutant |
|-------------------|-----------------------------|
| T39A              | 0.5                         |
| T35A              | 0.5                         |
| T26A              | 0.25                        |
| T31A              | 0.25                        |
| T30A              | 0.16                        |
| T33A              | 0.06                        |
| T35A/T39A         | 0.025                       |
| T31A/T39A         | 0.016                       |
| T30A/T39A         | 0.016                       |
| T33A/T39A         | 0.014                       |
| T30A/T31A         | 0.008                       |
| T30A/T35A         | 0.0027                      |

TABLE 4
The IC50 of the maxadilan threonine → alanine mutants with antagonist activity at the PAC1 receptor

| Antagonist threonine mutants | IC50 M65/IC50 mutant |
|-----------------------------|---------------------|
| T33A/T35A                   | 0.2                 |
| T35A/T33A/T39A              | 0.066               |
| T30A/T33A                   | 0.02                |
| T30A/T33A/T33A              | 0.0088              |
| T26A/T30A/T31A/T33A/T35A/T39A | 0.004              |
| T31A/T33A                   | 0.0033              |
| T26A/T30A/T31A/T33A         | 0.0028              |

gene. This construct (pTWIN1max) allowed maxadilan to be expressed with intein as a 32.5-kDa fusion protein (7.5 + 25 kDa), which after binding to a chitin column, was cleaved at pH 7.0, releasing maxadilan as a 7.5-kDa peptide.

Mutagenesis—All substitution and deletion mutants were made using the QuikChange site-directed mutagenesis method (Stratagene, La Jolla, CA). Complementary oligonucleotide primers 30–45 bases long were synthesized incorporating alanine codons (GCG/TGG/C/GCC/AGC/GCG) in the middle of the target sequence. As an example, the primers for the threonine mutant T33A are: 5'-CTC TCT GTA CAA ACA GCT GCA ACA TTC ACA TC-3' and 5'-GA TGT GAA TGT TGC AGC TGT TTG TAC AGA AG-3'. 200 ng of each of the primers and 40 ng of pTWIN1max as the template were combined in a 50-μl reaction mixture containing PfU DNA polymerase. The mixture was subjected to the following PCR conditions: 95 °C for 60 s, 18 cycles each of 95 °C for 50 s, 60 °C for 50 s and 68 °C for 8 min, and a final extension at 68 °C for 7 min. The product was incubated at 37 °C for 1 h with 1 μl of DpnI restriction enzyme to digest methylated template plasmid DNA. 1 μl of the reaction was transformed into competent Escherichia coli ER2566 cells. The resulting colonies were verified by DNA sequencing. The newly generated mutants were used as templates to create multiple substitutions in the same mutant.

Purification of Maxadilan—Purification of the maxadilan-intein fusion protein was based on the instructions provided by the manufacturer (New England Biolabs, Beverly, MA). The bacterial pellet was suspended in column buffer containing 20 mM Tris-HCl (pH 8.5), 500 mM NaCl, 0.1 mM EDTA, and 0.1% Triton X-100 and lysed in a French press. The suspension was centrifuged at 31,000 × g. The supernatant containing the fusion protein was passed over a chitin column pre-equilibrated with column buffer. The column was washed with column buffer extensively to remove unbound material and flushed with three column volumes of cleavage buffer containing 20 mM Tris-HCl (pH 7.0), 500 mM NaCl, and 0.1 mM EDTA. After incubating the column for 48 h, maxadilan was eluted with cleavage buffer and concentrated using Apollo 7 ultrafiltration concentrator devices (Orbital Biosciences, Topsfield, MA). Protein concentrations were measured by non-interfering protein assay (Geno Technology Inc., St. Louis, MO).

Assay of Peptide Activity in Melanophores—The maxadilan peptides were assayed for functional activity by measuring pigment dispersion in melanophores stably expressing the rat PAC1 receptor. Briefly, PAC1-expressing melanophores were plated in 96-well plates at a density of ~40,000 cells/well. Two hours prior to the addition of maxadilan or maxadilan mutants, the medium was changed and melanosomes were

16198 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 281 • NUMBER 24 • JUNE 16, 2006
aggregated with 2 nM melatonin, which targets the endogenous G linked melatonin receptor. Base-line readings were taken with a plate reader (Tecan, Maennedorf, Switzerland) at 620 nm to obtain the initial transmittance (T_i) values at time 0 before the addition of ligands. For the agonist assay, maxadilan and mutants were diluted from stock solutions and added to the wells in 10-fold dilutions in triplicate. The antagonist assay was performed by adding 10-fold dilutions of the mutants in the presence of 0.08 nM maxadilan. Final transmittance (T_f) readings were taken after 1 h. EC_{50} and IC_{50} values were generated as described previously (10).

RESULTS

The generation and functional investigation of 58 mutants of maxadilan were performed. Thirty-three substitution mutants affected selected charged residues and proline 43. Another nineteen mutants targeted the six threonine residues in the middle β-strand. Six other mutants are also described. All of the maxadilan mutants were produced as recombinant peptides in *E. coli* under similar conditions.

The mutants were investigated for agonist activity by measuring the transmittance changes as a result of pigment granule translocation in a PAC1-expressing melanophore line. This assay has the advantage of allowing for rapid functional evaluation of ligand-G protein-coupled receptor interaction. The inactive mutants were subsequently tested for antagonist activity using the same assay.

The mutant peptides affecting the charged residues and P43, compared with the wild-type maxadilan, have up to eight amino acid substitutions. All of these substitution mutants maintained agonist activity at the PAC1 receptor. The potency of the mutants is reported as a ratio between the EC_{50} of maxadilan and the EC_{50} of the mutants. The EC_{50} of maxadilan was ∼5 pm in the melanophore assay (Tables 1 and 2).

Alanine substitution mutants were engineered of threonines 26, 30, 31, 33, and 39, individually and in a variety of combinations, including all six residues. The large number of possible combinations precluded generating all of the potential combinations of mutants. However, the mutants that were generated, although limited in number, allow some conclusions to be drawn regarding the importance of these maxadilan β-strand threonine residues in PAC1 activation.

Each of the threonine → alanine mutations impaired the potency of the peptide. T33A was the least potent of the single substitution mutants (Table 3). Furthermore, seven multiple mutants lost their ability to activate PAC1. They were subsequently shown to be maxadilan antagonists with IC_{50} values ranging from 0.2 to 0.0028 relative to M65 (Table 4). These mutants were thus each less potent antagonists than M65, which in the melanophore assay, has an IC_{50} of 4 nM. A serine substitution mutant, replacing the threonines at positions 30, 31, 33, and 35 with the structurally similar amino acid serine, was found to have activity (EC_{50} = 60 pm) similar to wild-type maxadilan.

Deletion mutants Maxdel2 and Maxdel3, retaining the threonine-rich region absent in the antagonist M65, were inactive (Fig. 2). However, the deletion mutant Maxdel4, lacking the C-terminal lysine-rich residues, had a reduced activity with an EC_{50} value of 40 nM. The polyalanine mutant MaxPolyala1, with 11 alanines filling the space in the deletion mutant Maxdel3, was found to be active with an EC_{50} value of 0.3 nM. A similar polyalanine construct, MaxPolyala2, with 15 alanine residues replacing the deleted region in Maxdel2, was an antagonist (IC_{50} = 20 nM).

DISCUSSION

Of the 61 amino acids in maxadilan, natural variants in 21 residues have been reported. None of them affect activity as determined by relaxation of rabbit aortic rings. The four cysteine residues are conserved in all of these variants. Some amino acid variants are conservative, involving, for example, non-charged amino acids, such as serine and threonine at position 36. Other changes are not conservative, as in the case of valine or aspartic acid substitution at position 28. There is an overlap between several of the sites in the natural variants of maxadilan and the
alanine substitutions reported here (Fig. 1). However, only two of the variants have naturally occurring alanines at these residues, at positions 50 and 56 (9).

Because of its small size, it is understood that alanine substitution does not typically cause significant structural changes that would interfere with the activity of the mutated peptides. Resulting functional changes in the mutants would likely be due to altered ligand-receptor interaction. All of the single and multiple alanine substitution mutants generated in this study by targeting the charged residues or proline 43 maintained agonist activity at the PAC1 receptor (Tables 1 and 2). The least active of these mutants was K53A/K55A/K56A/K57A. It is likely that these lysine residues are responsible at least, in part, for the binding to the receptor. However, in contrast to the threonine → alanine mutants, none of the mutants affecting charged residues or proline 43 lost their activation properties, which makes them less likely to be involved in receptor activation.

Because deletion of the middle β-strand was previously shown to convert the molecule into an antagonist (8), we focused our investigation on this segment. We targeted, by alanine substitution, the six threonines in the 25–41 fragment of maxadilan, hypothesizing that these residues may be involved in hydrogen bonding to PAC1. All single mutants maintained agonist activity (Table 3). Targeting Thr-33 generated the least active single mutant (Fig. 3), which suggests that this residue may be the most important of the six threonines in receptor activation. Several double mutants maintained agonist properties (Table 3). They were less active than the corresponding single mutants, consistent with an additive effect of the mutations.

Three double mutants lost their activation properties completely. This observation, combined with the knowledge that deletion of 25–41 resulted in an antagonist, led us to test these mutants for activity as antagonists, which indeed turned out to be the case. Fig. 4 shows the antagonist activity of T31A/T33A in comparison to M65. Interestingly, all multiple mutants affecting more than two threonine residues became antagonists (Table 4). All of the antagonist mutants had Thr-33 substituted with alanine, which indicates once again that threonine at position 33 may be playing a major role in receptor activation. Thus, similar to M65, these antagonist mutants lose receptor activation properties but maintain binding properties at the PAC1 receptor. None of the threonine mutants lost both the activation and the binding properties.

These results provide additional evidence that supports the previous
deletion studies. It is likely that the middle β-strand of maxadilan is involved in receptor activation. This report identifies for the first time key specific maxadilan β-strand residues responsible for PAC1 activation. The results shown here reveal that all six threonine residues in the 25–41 maxadilan segment, with emphasis on Thr-33, are cooperatively involved in the activation of the PAC1 receptor. The interaction between the PAC1 receptor and the hydroxyl groups in these threonines is further supported by the serine substitution mutant (T30S/T31S/T33S/T35S), which retains activity.

The polyalanine mutant agonist MaxPolyala1 with 11 alanines filling the space of the deletion mutant MaxDel3 indicates that maxadilan activity also depends on the structural integrity of the region preceding the threonines. Activity appears to require a certain amount of space to be filled in the region between 15–25. In contrast, MaxPolyala2 was found to be an antagonist, perhaps because the missing Thr-26 and the neighboring residues are critical in this transition.

Taken together, we suggest a model for the interaction between maxadilan and the PAC1 receptor. It is possible that the C-terminal lysine residues of maxadilan initiate a non-activating binding interaction with the receptor. This initial interaction induces a conformational change in the receptor. The threonine residues of maxadilan, held in place by the disulfide bond between residues 14 and 51, can now fit into the newly generated pocket in the PAC1 receptor, leading to receptor activation (Fig. 5).

Based on the knowledge accumulated so far, maxadilan derivatives have the potential to be used as anti-hypertensives and immunomodulators. However, maxadilan is a peptide, limiting its usefulness as a therapeutic. There are no specific small molecular agonists at the PAC1 receptor. Understanding the molecular mechanisms of PAC1 activation may facilitate the future identification of such compounds. As a complement to these studies, it would be of interest to identify, via mutation analysis of the PAC1 receptor, which residues are involved in the interaction with maxadilan and whether or not those are the same residues that interact with PACAP.

Acknowledgment—We thank Phyllis Pereira for assistance with these studies.

REFERENCES
1. Lerner, E. A., Ribeiro, J. M., Nelson, R. J., and Lerner, M. R. (1991) J. Biol. Chem. 266, 11234–11236
2. Qureshi, A. A., Asahina, A., Ohnuma, M., Tajima, M., Granstein, R. D., and Lerner, E. A. (1996) Am. J. Trop. Med. Hyg. 54, 665–671
3. Morris, R. V., Shoemaker, C. B., David, J. R., Lanzaro, G. C., and Titus, R. G. (2001) J. Immunol. 167, 5226–5230
4. Milleron, R. S., Mutebi, J. P., Vallee, S., Montoya, A., Yin, H., Soong, L., and Lanzaro, G. C. (2004) Am. J. Trop. Med. Hyg. 70, 286–293
5. Moro, O., and Lerner, E. A. (1997) J. Biol. Chem. 272, 966–970
6. Pseugna, J. R., and Wank, S. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 90, 6345–6349
7. Vaudry, D., Gonzalez, B. J., Basille, M., Yon, L., Fournier, A., and Vaudry, H. (2000) Pharmacol. Rev. 52, 295–322
8. Moro, O., Wakita, K., Ohnuma, M., Denda, S., Lerner, E. A., and Tajima, M. (1999) J. Biol. Chem. 274, 23103–23110
9. Lanzaro, G. C., Lopes, A. H., Ribeiro, J. M., Shoemaker, C. B., Warburg, A., Soares, M., and Titus, R. G. (1999) Insect Biochem. Mol. Biol. 8, 267–273
10. Pereira, P., Reddy, V. B., Kounig, K., Bello, Y., and Lerner, E. (2002) Pigment Cell Res. 15, 461–466
11. Lerner, E. A., and Shoemaker, C. B. (1992) J. Biol. Chem. 267, 1062–1066