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The Vasoactive Peptide Maxadilan from Sand Fly Saliva Inhibits TNF-α and Induces IL-6 by Mouse Macrophages Through Interaction with the Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) Receptor[1]

Milena B. P. Soares,* Richard G. Titus,† Charles B. Shoemaker,* John R. David,* and Marcelo Bozza[2]*

Maxadilan is a vasodilatory peptide encoded by a gene cloned from *Lutzomyia longipalpis* salivary glands. In this study we investigated the effects of maxadilan on macrophage functions. Maxadilan treatment of LPS-stimulated BALB/c macrophages inhibited TNF-α release but increased IL-6. Further, it also induced IL-6 release in a dose-dependent manner from unstimulated macrophages. Maxadilan increased production of PGE_2, and the inhibition of TNF-α was completely abrogated by indomethacin. Others have recently shown that maxadilan is a selective agonist of the pituitary adenylate cyclase-activating polypeptide (PACAP) type I receptor. Treatment with the receptor antagonist PACAP 6–38 blocked maxadilan activities on macrophages. The natural endogenous ligand, PACAP 38, had the same effects as maxadilan on TNF-α and IL-6 production. Finally, in a dose- and time-dependent fashion, maxadilan induced the intracellular accumulation of cAMP in macrophages. Taken together, the results presented here indicate a modulatory effect of ligands of PACAP type I receptor on cytokine production by macrophages and suggest that activation of this receptor, with the subsequent elevation of intracellular cAMP in macrophages, could participate in a negative-feedback mechanism that controls certain inflammatory responses. ***The Journal of Immunology*****, 1998, 160: 1811–1816.**

In the course of a blood meal, hematophagous arthropods salivate into the host's skin, altering local homeostasis. Substances present in the saliva induce vasodilatation and prevent platelet aggregation, thus slowing blood coagulation (1). In addition, salivary gland lysates from phlebotomine sand flies, which are vectors for *Leishmania*, but not lysates from other bloodsucking arthropods, dramatically enhance the infectivity of *Leishmania major* for mice (2–4). Macrophages play a central role in the immune response to *Leishmania* infection, being both the host cell for the parasite and the effector cell responsible for its killing. Moreover, macrophages act as APCs and are an important source of several mediators, such as cytokines and nitric oxide (NO)[3] (5, 6). The enhancing effects of sand fly saliva on leishmaniasis are associated with its ability to selectively inhibit several macrophage functions, including Ag presentation (7), NO production, and the ability of macrophages to kill intracellular *L. major* (8).

Maxadilan was purified and its coding DNA cloned from the salivary glands of *Lutzomyia longipalpis* based on its ability to cause erythema (9, 10). The vasodilatory effect of maxadilan is endothelium independent and correlates with an increase of cAMP in smooth muscle cells (11). Maxadilan binds to membrane fractions of rabbit aorta and spleen, and to brain tissues from various species including human, bovine, rabbit, rat, and mouse (12). Maxadilan inhibits splenocyte proliferation and delayed-type hypersensitivity in mice (13), exacerbates murine cutaneous leishmaniasis, and inhibits killing of intracellular *L. major* by macrophages (our unpublished results). These studies indicate that maxadilan constitutes one of the immunomodulatory substances present in the sand fly saliva.

Moro and Lerner recently reported that maxadilan is a specific agonist of the pituitary adenylate cyclase activating polypeptide (PACAP) type I receptor (14). PACAP is a vasoactive neuropeptide with a plethora of biologic properties and is a member of a super family that includes secretin, glucagon, and vasoactive intestinal peptide (VIP) (15, 16). PACAP binds to at least two classes of seven-transmembrane G-coupled receptors: these are type I PACAP-preferring receptors and type II receptors, which also bind VIP with similar high affinity (15, 16). PACAP induces IL-6 release from folliculo-stellate cells from the rat pituitary (17), cortical astrocytes (18), and human placental choriocarcinoma cells (19). Moreover, PACAP inhibits proliferation of mitogen-stimulated splenocytes (20) and IL-10 production by T lymphocytes (21), suggesting a potential effect of this peptide in immune and inflammatory responses.

The present study was designed to examine the effects of maxadilan and PACAP on inflammatory cytokine production by macrophages and the mechanism by which maxadilan had its effects on macrophage functions.

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[3] Abbreviations used in this paper: NO, nitric oxide; PACAP, pituitary adenylate cyclase-activating polypeptide; CGRP, calcitonin gene-related peptide.

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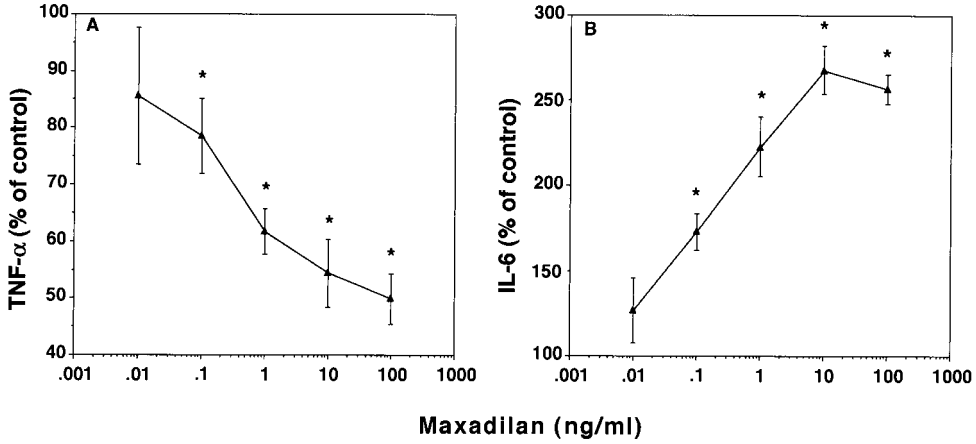

FIGURE 1. Maxadilan modulates TNF-α and IL-6 release by macrophages stimulated with LPS. BALB/c macrophages were preincubated for 2 h with different doses of maxadilan. An amount equal to 500ng/ml of LPS was then added to the macrophage monolayers and, 2 h (*A*) or 4 h (*B*) later, cell-free supernatants were collected. TNF-α and IL-6 contents were determined by ELISA. Values are shown as percent of LPS control from three independent experiments, expressed as mean ± SEM. * $p < 0.05$ compared with LPS alone. Levels of TNF-α in LPS control cultures varied from 100 pg/ml to 3 ng/ml, and of IL-6, from 1 to 6 ng/ml.

Materials and Methods

Mice

Animals used were: 6-wk-old female BALB/c (Charles River Laboratories, Boston, MA); 6-wk-old male C3H/HeJ (The Jackson Laboratory, Bar Harbor, ME). The animals were housed at the Department of Tropical Public Health, Harvard School of Public Health animal facilities.

Reagents

Reagents were LPS from *Escherichia coli* serotype 0111: B4, and thioglycollate and indomethacin (Sigma Chemical, St. Louis, MO); DMEM, HBSS, L-glutamine, HEPES, sodium pyruvate, penicillin, and streptomycin (Life Technologies/Life Technologies, Gaithersburg, MD); FCS (HyClone Laboratories, Logan, UT); synthetic rat PACAP 38 and PACAP 6–38 (Peninsula Laboratories, Belmont, CA); and neutralizing anti-IL-6 and anti-IL-I0 (PharMingen, San Diego CA).

The coding DNA of mature maxadilan as reported by Lerner and Shoemaker (10) was amplified by PCR and introduced into pRSET from InVitroGen (Carlsbad, CA) to generate the expression vector XI13–3. An inactive mutant version of the same maxadilan-coding DNA (cysteine 51 to alanine) was fortuitously generated by the same PCR reaction and introduced into pRSET to generate XI13–1 (our unpublished results). *E. coli* harboring the plasmids were induced for expression as recommended by InVitroGen, to produce recombinant maxadilan having amino terminal fusion partners that contain a hexa-histidine to facilitate purification. The recombinant fusion proteins were purified from inclusion bodies as described (22). All the reagents used in culture, including recombinant and synthetic peptides, contained <10 pg/ml of endotoxin, measured by *Limulus* assay from BioWhittaker (Walkersville, MD).

Synthetic maxadilan was prepared by the Biopolymers Laboratory in the Harvard Medical School. The amino acid sequence was based on the predicted sequence of mature, secreted maxadilan obtained by sequencing a maxadilan cDNA isolated from a sand fly obtained from Belo Horizonte, Brazil (our unpublished results).

Macrophage cultures

To obtain peritoneal exudate macrophages, mice were injected with 2 ml of sterile 3% thioglycollate in saline i.p., and after 4 days peritoneal lavage was performed using 10 ml of cold HBSS. After two washes with HBSS, the cells were resuspended in DMEM supplemented with 10% FCS and plated in 24-well tissue culture plates at 2×10^6 per well in 1 ml. After 2 h of incubation at 37°C, nonadherent cells were removed by two washes with culture medium. This adherent population is ≥ 95% Mac-1^+ cells (23). Macrophages were then further incubated and treated as described in the text or figure legends, and cell-free supernatants were frozen for subsequent cytokine and PGE_2 measurement.

Cytokine and PGE_2 determinations

Supernatants were tested for TNF-α and IL-6 using a sandwich ELISA according to the manufacturer's directions. Abs for TNF-α and IL-6 ELISAs, and recombinant mouse TNF-α and IL-6, were purchased from PharMingen. PGE_2 was measured using an EIA kit from Cayman Chemical Co. (Ann Arbor, MI) according to the manufacturer's directions.

Measurement of intracellular cAMP

Macrophages were plated as described above. After incubation with isobutylmethylxanthine (1 mM) for 30 min, maxadilan was added to the cultures for 2, 15, 30, 45, or 60 min. The medium was then aspirated and the reaction was stopped by adding 1 ml of cold ethanol to the wells. Samples were centrifuged and frozen until assayed. cAMP contents were determined using an EIA kit from Cayman Chemical Co. according to the manufacturer's directions.

Statistical analysis

Data were analyzed for significance using Student's *t* test. Data with $p \leq 0.05$ were considered significant.

Results

Maxadilan modulates cytokine production by LPS-stimulated macrophages

It has been shown that sand fly saliva affects macrophages, decreasing Ag presentation (7), NO production, and *Leishmania* killing (8). Therefore, we investigated whether maxadilan would affect cytokine production by macrophages treated with LPS, a potent and well-studied macrophage activator. Thioglycollate-elicited peritoneal macrophages were pretreated with increasing concentrations of maxadilan for 2 h, followed by LPS treatment (500 ng/ml) for an additional 2 or 4 h, and the media was assayed for TNF-α and IL-6 secretion, respectively. In a dose-dependent fashion, maxadilan markedly decreased LPS-induced TNF-α secretion (Fig. 1*A*). Conversely, IL-6 production was markedly enhanced by maxadilan, also in a dose-dependent manner (Fig. 1*B*). The maximum inhibition of TNF-α production (about 50%) and enhancement of IL-6 levels (two- to threefold) was observed at concentrations of 1 to 10 ng of maxadilan/ml (0.1 to 1 nM).

Further, we investigated whether maxadilan was capable of inducing the release of IL-6 from macrophages without LPS stimulation. In a dose-dependent fashion, recombinant maxadilan induced IL-6 release from thioglycollate-elicited peritoneal macrophages (Fig. 2). Since the recombinant maxadilan that we used was made in *E. coli*, we determined whether the following additional forms of maxadilan would induce IL-6 production by macrophages: 1) a mutated recombinant form of maxadilan that lacks vasodilatory activity, but which is produced in *E. coli* in a

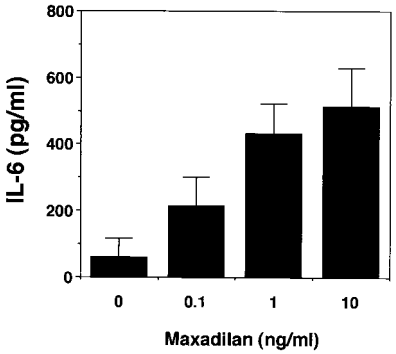

FIGURE 2. Maxadilan induces IL-6 release by normal macrophages. BALB/c macrophages were incubated for 6 h with the indicated doses of recombinant maxadilan. Supernatants were collected and tested for IL-6 by ELISA. Data represent the mean ± SEM of three separate experiments.

fashion identical to functional maxadilan, and 2) a synthetic form of maxadilan. While the mutated maxadilan did not induce IL-6 production by macrophages, the synthetic maxadilan induced IL-6 production to an extent similar to bioactive recombinant maxadilan (data not shown). In addition, to further rule out the possibility that LPS contamination contributed to our observations, we tested whether maxadilan could induce IL-6 production by macrophages from C3H/HeJ mice, which are endotoxin-resistant (24). We found that maxadilan, in a dose-dependent manner, also induced IL-6 production by C3H/HeJ macrophages (data not shown).

Maxadilan inhibition of LPS-induced TNF-α release is dependent on PG

Several studies demonstrated the modulatory effects of PGE_2 and IL-10 on macrophage activation, including the inhibition of TNF-α production (25–28). Moreover, IL-6 can cause a reduction in LPS-induced TNF-α release from a human monocytic cell line, from human whole blood, and in vivo (29). Since these three mediators are produced by macrophages activated with LPS, we determined the role of PGE_2, IL-10, and IL-6 in the maxadilan inhibition of LPS-induced TNF-α production. Treatment with indomethacin completely abrogated the inhibition by maxadilan of LPS-induced TNF-α release (Fig. 3). On the other hand, maxadilan was able to inhibit TNF-α in the presence of neutralizing anti-IL-10 Abs (Fig. 3, compare *bars 6* and *7*) or anti-IL-6 (data not shown).

Because PGE_2 is the main PG that inhibits TNF-α production (25) and also a major target of indomethacin, we measured PGE_2 production by macrophages stimulated with LPS in the presence of maxadilan. The addition of maxadilan consistently led to a dose-dependent increase in PGE_2 release (Fig. 4).

Maxadilan effects on cytokine production by macrophages are mediated by activation of the PACAP receptor

It has been recently shown that maxadilan is a selective agonist of the PACAP type I receptor (14). Similar to many other vasoactive neuropeptides, PACAP mediates a range of biologic activities, including the induction of IL-6 release from several cell types (17–19). To test whether the effects we observed on IL-6 and TNF-α production were mediated through interaction of maxadilan with the PACAP receptor, we investigated whether the PACAP-competing receptor antagonist PACAP 6–38 blocked the effects of maxadilan on cytokine production. The addition of 1 μg/ml of PACAP 6–38 completely blocked the inhibitory effect of 10 ng/ml of maxadilan on LPS-induced TNF-α release (Fig. 5*A*). Likewise, IL-6 production induced by maxadilan was also abrogated (Fig. 5*B*), indicating that both effects of maxadilan are mediated by signaling through the PACAP receptor. The addition of the same amount of CGRP antagonist (CGRP 8–37) did not alter the effects of maxadilan on IL-6 and TNF-α secretion (data not shown)

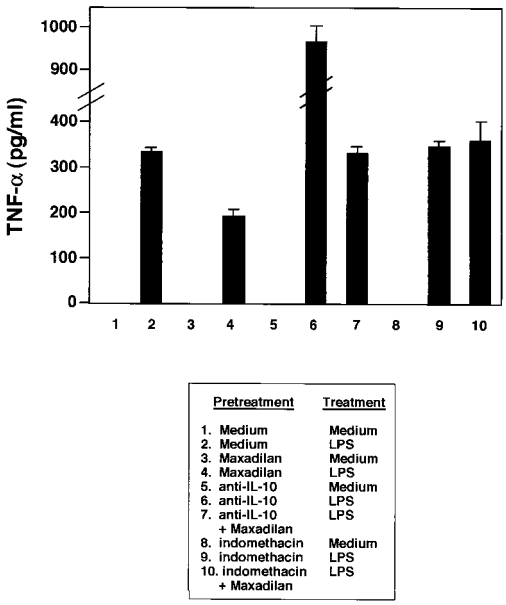

FIGURE 3. The inhibition of TNF-α by maxadilan on LPS-stimulated macrophages is blocked by indomethacin but not by anti IL-10. Macrophages were plated as described in *Materials and Methods*. After 2 h preincubation in the presence or in the absence of anti-IL-10 (5 μg/ml), indomethacin (1 μg/ml), and maxadilan (10 ng/ml), LPS (500 ng/ml) was added for an additional 4 h. Supernatants were collected and tested for TNF-α by ELISA. Results shown are from one representative of four independent experiments performed. Values are expressed as mean ± SEM of four determinations.

PACAP 38 inhibits TNF-α release and induces IL-6 production from LPS-stimulated macrophages

The results obtained with the PACAP receptor antagonist (Fig. 4) suggested that PACAP might also modulate TNF-α and IL-6 release from macrophages. PACAP exists in two amidated forms: PACAP 38 and PACAP 27 (30, 31). Since PACAP 38 binds to the PACAP type I receptor with higher affinity compared with PACAP

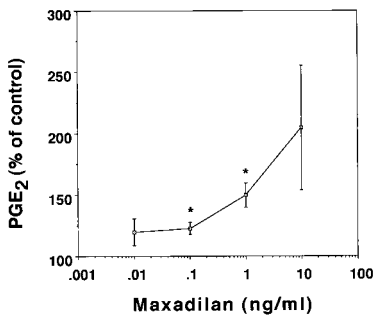

FIGURE 4. Maxadilan increases PGE_2 from macrophages stimulated by LPS. BALB/c macrophages were preincubated for 2 h with different doses of maxadilan. An amount equal to 500 ng/ml of LPS was then added to the macrophage monolayers, and, 2 h later, the cell-free supernatants were collected and tested for the presence of PGE_2 by enzyme immunoassay. Values are shown as percent of LPS control from four independent experiments, expressed as mean ± SEM. * $p < 0.05$ compared with LPS alone. Levels of PGE_2 in LPS control cultures varied from 100 pg/ml to 1.5 ng/ml.

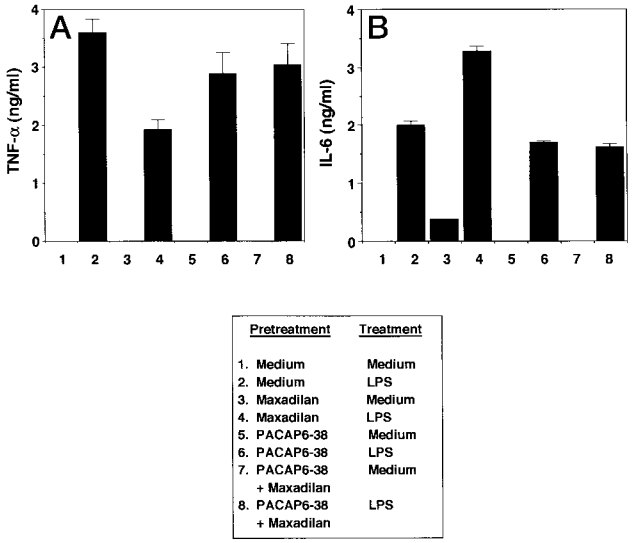

FIGURE 5. PACAP 6–38 blocks the effects of maxadilan on macrophages. BALB/c macrophages were preincubated for 2 h with maxadilan (10 ng/ml) in the presence or absence of PACAP 6–38 (1 μg/ml). LPS (500 ng/ml) was then added and the supernatants were collected after 2 or 4 h for TNF-α (*A*) and IL-6 (*B*), respectively, and tested by ELISA. Results shown are from one representative of five independent experiments performed. Values represent mean ± SD of four determinations.

27 (32) and constitutes the majority of the immunoreactive PACAP in several tissues (16), we decided to use this form in the present study. Preincubation of macrophages with 10 ng/ml of PACAP 38 led to a reduction of LPS-induced TNF-α production similar to that observed with maxadilan (Table I). Finally, PACAP 38 also induced IL-6 release from macrophages and increased LPS-induced IL-6 release (Table I).

Maxadilan increases intracellular levels of cAMP in macrophages

Activation of the PACAP type I receptor by it ligands markedly increases the level intracellular cAMP in several cell types (14, 17–19). Furthermore, the vasodilatory effect of maxadilan correlates with an increase of cAMP (11). Since elevation of intracellular cAMP modulates several macrophage functions, we investigated the effects of maxadilan on this second messenger. Figure 6 shows a time course and a dose response for maxadilan induction of intracellular cAMP in mouse macrophages. Within minutes after treating with 10 ng/ml of maxadilan, the levels of intracellular cAMP increased severalfold (Fig. 6*A*). Moreover, the effect of maxadilan on cAMP levels was a dose-dependent phenomenon (Fig. 6*B*).

Table I. *PACAP 38 modulates cytokine release by macrophages*[a]

Experiment	1	2	3	4
TNF-α (ng/ml)				
Medium	0	0	0	0
LPS	0.34	0.16	1.13	0.55
PACAP	0	0	0	0
PACAP + LPS	0.13	0.04	0.70	0.20
IL-6 (ng/ml)				
Medium	0	0	0	0
LPS	1.99	5.36	1.61	2.96
PACAP	0.55	1.08	0.78	1.55
PACAP + LPS	3.05	6.38	1.92	3.49

[a] BALB/c macrophages were preincubated for 2 h in the presence or absence of 10 ng/ml of PACAP 38. LPS (500 ng/ml) was added, and supernatants were collected after 4 h for TNF-α and IL-6 and tested by ELISA. Using the Mann-Whitney test, the decrease in TNF-α and increase in IL-6 when PACAP is preincubated with macrophages before adding LPS is significant, $p < 0.05$.

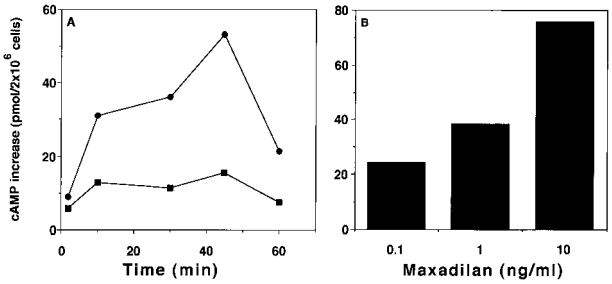

FIGURE 6. Maxadilan dramatically enhances the level of intracellular cAMP in macrophages. *A*, Time course of cAMP accumulation. BALB/c macrophages were incubated with 10 ng/ml of maxadilan for different times, and the levels of intracellular cAMP were determined (techniques in *Material and Methods*). *B*, Dose response of cAMP accumulation. BALB/c macrophages were incubated with different doses of maxadilan for 15 min, and the levels of intracellular cAMP were determined. The figure is representative of three independent experiments.

Discussion

The results presented here indicate that maxadilan augments IL-6 production while it inhibits TNF-α production by macrophages through interaction with the PACAP receptor. These conclusions were reached using multiple corroborating experimental approaches. Both recombinant (Fig. 2) as well as synthetic maxadilan had similar effects on IL-6 secretion by macrophages. Using LPS-stimulated macrophages, maxadilan again augmented IL-6 production while it inhibited TNF-α secretion (Fig. 1). These effects on macrophages were mediated by maxadilan itself (and not another component of our recombinant maxadilan preparation, such as LPS), since treating macrophages with a mutated recombinant maxadilan did not modulate cytokine release, and maxadilan increased IL-6 production even with macrophages obtained from LPS-unresponsive C3H/HeJ mice (data not shown). Maxadilan exerted its effects on macrophages through interaction with the PACAP receptor since the addition of PACAP receptor antagonist, PACAP 6–38, completely blocked the effects of maxadilan on IL-6, as well as TNF-α release by macrophages (Fig. 5). Finally, synthetic PACAP 38, the endogenous ligand of the PACAP receptor, modulated cytokine production by macrophages in a manner similar to maxadilan (Table I).

To determine the mechanisms by which maxadilan exerts its inhibitory effects on TNF-α release from LPS-stimulated macrophages, we treated the macrophage cultures with indomethacin, neutralizing anti-IL-10 and neutralizing anti-IL-6 Abs. Indomethacin completely abrogated the inhibitory effect of maxadilan on TNF-α production by macrophages challenged with LPS (Fig. 3), indicating that PGs are involved in the maxadilan modulation of TNF-α production by macrophages. In fact, treatment of macrophages with maxadilan and LPS led to an increase of PGE_2 in the supernatants compare with the controls (Fig. 4). It has been reported that IL-10 participates in the deactivation of LPS-stimulated macrophages by PGE_2 (33). In our experiments, we found that maxadilan was able to inhibit TNF-α by greater than 50% in the presence of anti-IL-10. Treatment with anti-IL-6 had no effect on TNF-α inhibition by maxadilan. Finally, indomethacin or anti-IL-10 treatment did not interfere with maxadilan-induction of IL-6 (data not shown). In opposition to the effect of indomethacin on

maxadilan inhibition of TNF-α release from macrophages, the erythema or the vasodilatation of rabbit aorta rings induced by maxadilan was not blocked by indomethacin (34, 11). Taken together, these results indicate a dissociation in the involvement of PG in the effects of maxadilan on TNF-α and IL-6 production by macrophages, as well as in the relaxing activity on smooth muscle cells. The mechanism by which maxadilan increases PGE_2 synthesis remains to be determined.

Inflammatory mediators produced by macrophages are thought to play a role in the etiology of a variety of pathologic conditions, such as bacterial, protozoan, and fungal infections, as well as acute and chronic inflammatory reactions. We originally reported that sand fly saliva exacerbated infection with *L. major* in mice and that the target cell for the saliva seemed to be macrophages (7, 8). The results presented here extend these observations by showing that a single protein derived from sand fly saliva, maxadilan, modulates the production of two proinflammatory cytokines by macrophages, IL-6 and TNF-α, and does so through interaction with the PACAP receptor. We have since shown that maxadilan also exacerbates *L. major* infection in mice and inhibits intracellular killing of the parasite in macrophages (unpublished results). Thus, maxadilan constitutes at least one of the immunomodulatory substances present in sand fly saliva.

Regulation of IL-6 production is complex, being mediated by several signal transduction pathways and involving a number of second messengers and protein kinases, including intracellular Ca^+, cAMP, and protein kinase C (35–38). The ability of both PACAP and maxadilan to stimulate IL-6 production is associated with increased levels of intracellular cAMP (17–19, Figs. 1*B*, 2, 6). Indeed, treating with the protein kinase A inhibitor H89 reduced PACAP-mediated induction of IL-6 secretion by pituitary cells (17). However, PACAP also activates multiple intracellular signaling pathways including protein kinase A, phospholipase C, and protein kinase C translocation (30, 39–41). These results suggest that the effects of maxadilan and PACAP 38 on IL-6 release by macrophages are at least in part mediated by the second messenger cAMP, but that it is likely that both maxadilan and PACAP 38 mediate their effects by triggering more than one intracellular signaling pathway.

cAMP may also be involved in the mechanism by which maxadilan inhibits TNF-α production by macrophages (Fig. 1*A*). Several reports indicate that elevation of intracellular cAMP in macrophages has an inhibitory effect on LPS-induced TNF-α production (25, 33, 42, 43). Current experiments are investigating further the mechanisms by which maxadilan modulates macrophage function, as well as the effects of this peptide on inflammatory reactions in vivo.

Previous studies have demonstrated that the vasoactive neuropeptide substance P stimulates secretion of TNF-α, IL-1, and IL-6 from macrophages (44), and enhances secretion of IL-1 from LPS-stimulated microglia (45), and TNF-α by LPS-stimulated neuroglial cells (46). To our knowledge, these results constitute the first demonstration that ligands of the PACAP type I receptor, such as maxadilan and PACAP 38, have an effect opposite from that of substance P on TNF-α release by macrophages. Furthermore, we have observed that another vasoactive neuropeptide, calcitonin gene related peptide (CGRP), also inhibits LPS-induced TNF-α production by macrophages in a manner similar to that of maxadilan or PACAP 38 and in vivo protects mice against a lethal dose of LPS (our unpublished observations). Therefore, it is tempting to speculate that there exist two classes of vasoactive neuropeptides that have opposite effects on TNF-α secretion by macrophages. As a result, these neuropeptides might play a critical role in counter-regulating the development of inflammatory and immune responses.

In conclusion, the results presented here suggest that signaling through the PACAP type I receptor on macrophages, with a subsequent rise in intracellular levels of intracellular cAMP, could be involved in blocking inflammatory responses as well as increasing susceptibility to intracellular pathogens. Specifically, these results suggest that the ability of sand fly saliva to exacerbate infection with *L. major* lies at least in part in its ability to mimic the action of neuropeptides such as PACAP on cytokine release by macrophages. During evolution, sand flies presumably developed anti-inflammatory proteins in their saliva to prevent the vertebrate host from becoming sensitized to the proteins of their saliva. Inadvertently, these same immunosuppressive proteins appear to promote infection with the pathogens sand flies transmit. In addition to sand flies, immunomodulatory factors have been described in the saliva of a number of blood-sucking arthropods including mosquitoes, black flies, and ticks (47–51). Moreover, tick saliva can enhance virus infectivity (52). It will be interesting to determine whether immunomodulators in the saliva of arthropods other than sand flies will also employ neuropeptide mimicry as one their mechanism of action.

Acknowledgments

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