Salivary Gland Homogenates of *Lutzomyia longipalpis* and Its Vasodilatory Peptide Maxadilan Cause Plasma Leakage via PAC1 Receptor Activation

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Key Words

Arteriolar dilation • *Lutzomyia longipalpis* • Maxadilan • PAC1 receptor • Pituitary adenylate cyclase-activating peptide • Plasma leakage • Postcapillary venules • Salivary glands

Abstract

Objectives: Experiments were designed to determine if salivary gland homogenates (SGH) of the sand fly *Lutzomyia longipalpis*, the vasodilatory peptides maxadilan and pituitary adenylate cyclase-activating peptide (PACAP-38) may cause plasma leakage and to what extent these effects could be due to PAC1 receptor stimulation. **Methods:** Using FITC-dextran as a plasma marker, intravital microscopy of the hamster cheek pouch (HCP) and a digital camera were used to assess arteriolar diameter and fluorescence of a selected area (5 mm²) representative of the HCP microcirculation. **Results:** Cheek pouches prepared for intravital microscopy and exposed to topical application of SGH, maxadilan or PACAP-38 developed maximal dilation of arterioles in the range of 20–60 µm within 10 min, and this effect lasted for 30–90 min. The increase in fluorescence intensity induced by each of these compounds was due to plasma leakage from postcapillary venules. The mutant peptide of maxadilan (M-65), a PAC1 re-

ceptor antagonist, inhibited both dilation and plasma leakage induced by SGH or maxadilan. Plasma leakage induced by SGH was modestly inhibited by the bradykinin B₂ receptor antagonist HOE-140, but not by the antihistamine mepyramine or the nitric oxide synthase inhibitor L-NA. **Conclusions:** SGH of *L. longipalpis* and its vasodilatory peptide maxadilan caused long-lasting arteriolar dilation and plasma leakage in the cheek pouch via PAC1 receptor activation.

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Introduction

Saliva of blood-feeding arthropods plays an important role in food ingestion as well as pathogen transmission. Saliva injected into vertebrate hosts contains active molecules that not only counteract vertebrate hemostasis, allowing the arthropods to feed successfully [1], but also modulate host immune responses [2]. Among these arthropods, phlebotomine sand flies are the vectors of *Leishmania* parasites, the causal agents of leishmaniasis, a disease affecting 2 million people annually in 88 countries [3]. *Lutzomyia longipalpis*, the New World vector of *Leishmania chagasi*, is the main vector of the visceral form of leishmaniasis, being fatal if untreated [4]. *Leish-*

mania are transmitted when female sand flies salivate in the biting site while obtaining their blood meal. Saliva, which contains a vast array of pharmacologically active substances allowing blood feeding, may also enhance or facilitate infectivity of parasites by downmodulating host immune responses [5–7]. Several studies have shown that salivary gland lysates, or its most active vasodilator component, maxadilan, may enhance infectivity of *L. chagasi*, *L. donovani*, *L. major*, *L. braziliensis* and *L. amazonensis* [8–12]. Among the repertoire of *L. longipalpis* salivary proteins, the potent vasodilator named maxadilan was characterized and cloned [13–15]. Cloning of the maxadilan gene revealed that the primary sequence of this polypeptide has no homology to that of pituitary adenylate cyclase-activating peptide (PACAP), despite the fact that maxadilan is an agonist of the PAC1 receptor, one of the three receptors of PACAP [16, 17]. PACAP is a member of the secretin/glucagon/vasoactive intestinal peptide superfamily of peptides, originally isolated from the ovine brain in 1989 [18]. Two biologically active forms, PACAP-38 and PACAP-27, have been derived from proteolytic processing of a single 176-amino acid precursor [19, 20]. More recently, Reddy et al. [21] engineered several mutants of the 61-amino acid vasodilator peptide maxadilan and then proceeded to characterize their binding specificity for the PAC1 receptor. Based on this analysis, some mutants were characterized as PAC1 receptor antagonists, e.g. the 44-amino acid polypeptide called M-65 [21]. Studies of the biological effects of PACAP revealed that it enhanced the permeability-inducing effects of bradykinin in the skin of rabbits [22, 23] and rats [24]. Further, in the human nose, PACAP-38 caused extravasation of plasma albumin [25]. It may thus be expected that PACAP and maxadilan could have the same effects in the hamster cheek pouch (HCP), a skin-like tissue without glands and hair follicles.

Among the myriad of vasoactive and immunomodulatory compounds found in sand fly salivary glands [26], maxadilan and adenosine injection can provoke systemic effects through the induction of arteriolar vasodilation and the stimulation of pro- or anti-inflammatory mediators, e.g. TNF- α , IL-6 or IL-10 [27–29].

There is much experimental evidence that vasodilation will only enhance extravasation of large molecules (plasma leakage) if there is a concomitant stimulation of postcapillary endothelial cells by the vasodilator or by some other agent. Although sand fly-injected parasites may increase their infectivity at the expense of the vasodilating effects of maxadilan [12], it remains to be determined if this vasoactive compound may independently

promote plasma leakage via activation of PAC-1 receptors expressed by neutrophils and monocytes [30, 31].

Here, we used HCP as a model to investigate if salivary gland homogenate (SGH) may evoke arteriolar dilation and extravasation of plasma from postcapillary venules, and then characterized the vasoactive responses induced by maxadilan and PACAP-38. Apart from their predicted vasodilatory effects, SGH, maxadilan and PACAP-38 were able to independently evoke plasma leakage in the cheek pouch. The finding that the M-65 mutant of maxadilan reduced both vasodilation and plasma leakage induced by SGH or maxadilan suggests that their vasoactive and pro-inflammatory effects are at least partially mediated by activation of PAC1 receptors.

Animals and Methods

Sand Flies and Preparation of SGH

L. longipalpis sand flies were obtained from the Camará colony maintained at the Department of Entomology, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil. Salivary glands from female sand flies, 3–5 days old, non-blood fed, were dissected out in cold phosphate-buffered saline (pH 7.0). Glands were sonicated for 3 cycles at 35% amplification (Ultrasonic processor 750 W), centrifuged at 8,000 rpm and supernatants stored at -80°C until thawed for use.

Intravital Digital Microscopy of the HCP

Hamsters were anesthetized by intraperitoneal injection of sodium pentobarbital supplemented with intravenous α -chloralose (2.5% W/V in saline solution) through a femoral vein catheter. A tracheal cannula (PE 190) was inserted to facilitate spontaneous breathing, and the body temperature was maintained at 37°C using a heating pad connected to a rectal thermistor probe. HCP was prepared and used for intravital microscopy as previously reported [32–34]. Briefly, the cheek pouch was everted and mounted on a microscope stage, and an area of about 1 cm^2 was prepared for intravital microscopy observations of the microcirculation. Thirty minutes after completing the preparation, fluorescein-labeled dextran (100 mg/kg body weight; FITC-dextran, molecular weight = 150 kDa; TdB Consultancy, Uppsala, Sweden) was intravenously injected as a macromolecular tracer. HCP were continuously superfused with a HEPES-bicarbonate-buffered saline solution (pH 7.4; composition in mM: 110.0 NaCl, 4.7 KCl, 2.0 CaCl_2 , 1.2 MgSO_4 , 18.0 NaHCO_3 , 15.39 HEPES and 14.61 Na HEPES) at a constant rate of 5 ml/min. The superfusion solution was bubbled with a 5% CO_2 -95% N_2 mixture to maintain a low physiological oxygen level around the cheek pouch. A heater was adjusted to maintain the superfusion temperature at 35°C . HCP microcirculation was assessed using an Axioskop 40 microscope with a $\times 4$ objective and $\times 10$ oculars (Zeiss, Oberkochen, Germany) equipped with appropriate filters (490/520 nm) for observations of epilluminescence fluorescence. Images were captured with a digital camera (AxioCamHRc; Zeiss) and processed with AxioVision software (AxioVision 4.4; Zeiss). Each AxioCamHRc image

of a 5-mm² area consisted of 1,388 × 1,040 pixels. Image width was 1,388 pixels or 2,500 μm and image height was 1,040 pixels or 2,000 μm. Each recorded image in the computer consisted of 8.37 MB and was stored concomitant with technical data, and date and hour of exposure. A typical experiment required 200–300 MB. AxioVision 4.4 was used for measurements of arteriolar diameter and total fluorescence in a representative rectangular area (5 mm²) of the prepared HCP (~1 cm²), with one or two arterioles ranging from 20 to 60 μm in diameter being suitable for measurements. For group comparisons, the diameter at the end of the 30-min control period (time 0) was set to 100%. Following intravenous injection of FITC-dextran, images of the microcirculation (5 mm²) were recorded at 5-min intervals during the entire experimental period and stored in a computer. A standardized exposure time (100 ms) was used in all experiments, and background fluorescence was assessed before FITC-dextran injection with no light source activated (~60 fluorescence units) and with the UV light (275 ± 70 fluorescence units, n = 68). The standardized dose of FITC-dextran (100 mg/kg body weight) resulted in a total fluorescence of 2,238 ± 942 units (n = 68) in the 5-mm² area 30 min after intravenous injection. The recorded 30-min fluorescence in each experiment was adjusted to 2,000 fluorescence units for statistical reasons. The original method for studies on microvascular permeability changes in the HCP that implied counting of sites (leaks) with extravasation of FITC-dextran in postcapillary venules [32] has been verified in several studies by measuring FITC-dextran concentrations of the superfusion buffer [35–40]. In order to validate our present modification of the original method involving a digital camera connected to a microscope (AxioVision and AxioCam HRC) for fluorescence measurements in a rectangular area (5 mm²) of the HCP (~1 cm²), the superfusion solution was sampled every 5 min during the hour following the 2-min application of histamine (10⁻⁵ M). FITC-dextran concentrations of the superfusion solution were determined by fluorimetry (Hitachi FH 4500; Hitachi, Tokyo, Japan).

After FITC injection (100 mg/kg body weight) and an initial 30-min control period to establish normal blood flow and vascular permeability conditions, superfusion of the HCP was interrupted, and agents diluted to appropriate testing concentrations in a volume of 250 μl saline were applied on the dissected HCP area during 9 min until superfusion was restarted. This procedure has been found to cause no significant alterations in arteriolar diameter or fluorescence upon restart of the interrupted superfusion. SGH equal to half (SGH-0.5) or one salivary gland (SGH-1), according to the dose used by Morris et al. [41] in 2001, were topically applied on the HCP for 9 min after interruption of superfusion. The concentrations of maxadilan (33 and 67 nM) were determined in pilot experiments, with vasodilation and plasma leakage being similar to the effects of SGH. The maxadilan mutant M-65 was applied at a concentration of 8 μM (120 times the concentration of 67 nM maxadilan) and PACAP-38 was applied at 50 nM in 250 μl of saline. Tested compounds from stock solutions kept on ice were immediately diluted in 250 μl of saline prior to their application on the HCP.

Thirteen groups of male hamsters (68 hamsters in toto) were studied: group 1 (control group; n = 6) – 250 μl PBS at 0 min; group 2 (n = 6) – SGH-0.5; group 3 (n = 5) – SGH-1, group 4 (n = 5) – 33 nM maxadilan in PBS; group 5 (n = 7) – 67 nM maxadilan in PBS; group 6 (n = 5) – local treatment first with 8 μM M-65 and then with M-65 + maxadilan; group 7 (n = 5) – 8 μM M-65 + SGH-

1 followed by SGH-1; group 8 (n = 5) – 250 μl of a 50 nM PACAP-38 solution in saline; group 9 (n = 4) – treatment with 4 μM of PACAP6-38, a competitive PAC1 receptor antagonist, 2 min prior to the application of 67 nM maxadilan or 250 μl PBS (n = 3, control), and group 10 (n = 3) – 8 μM M-65 given 2 min prior to PACAP-38 application (50 nM). Three further groups were studied to evaluate the role of bradykinin, histamine and nitric oxide in SGH-induced effects: group 11 (n = 5) – treatment with the bradykinin-2-receptor antagonist HOE-140 (0.5 μM) 5 min prior to SGH-0.5 application; group 12 (n = 5) – treatment with the antihistamine mepyramine (25 μM) prior to SGH-0.5 application, and group 13 (n = 4) – treatment with L-nitro-arginine (L-NA, 10 μM) 5 min prior to SGH-0.5. Recombinant maxadilan and the maxadilan antagonist M-65 were obtained as described [21]. PACAP-38 was from Sigma (St. Louis, Mo., USA), EUA and PACAP6-38 were from Bachem (Bubendorf, Switzerland).

Statistical Analyses

Statistical evaluation was performed with ANOVA followed by Student's t test (between groups) and paired t test (within groups). A p value of 0.05 or less was considered to indicate a statistically significant difference. Data are presented as means ± SEM.

Results

Fluorescence Increase in the HCP and in the Superfusion

Parallel measurements of histamine-induced plasma leakage with the digital camera and of FITC-dextran concentrations of the superfusion fluid leaving from the microscope stage with the mounted cheek pouch showed that measurements in a 5-mm² area of the HCP microcirculation were representative of changes in the entire HCP preparation, as reflected by FITC-dextran concentrations in the superfusion fluid (fig. 1, 2). The maximal response to 10⁻⁵ M histamine for 2 min detected by the digital camera occurred approximately 10 min earlier than the peak value of FITC-dextran (expressed in pg·ml⁻¹). A linear regression analysis after adjusting maximal response for this time shift revealed a highly significant linear correlation between fluorescence of the 5-mm² area and the FITC-dextran concentrations (n = 12, r = 0.973 and p = 0.00001). Thus, measurements of fluorescence in a restricted area of the HCP reflected histamine-induced vascular permeability changes in the entire HCP preparation.

Arteriolar Dilation Induced by SGH and Maxadilan

At end of the initial 30-min control period, arteriolar diameter varied from 38.9 ± 16.9 μm (mean ± SD) in the mepyramine group to 51.8 ± 11.5 μm in the 67-nM maxadilan group. There were no statistically significant differences in these diameters between groups (fig. 4; p > 0.1).

Fig. 1. Four images of the 5-mm² rectangular HCP areas that were used for fluorescence measurements (fig. 2) prior to (a), and 1 (b), 4 (c) and 30 min (d) after histamine application.

Fig. 2. Fluorescence in a 5-mm² rectangular HCP area (fig. 1) and FITC-dextran concentrations in the superfusion of the same HCP (pg/ml superfusion). Histamine (10^{-5} M) was applied for 2 min, resulting in parallel increases in fluorescence of the cheek pouch and of the FITC-dextran concentration in the superfusion fluid leaving from the microscope stage with the mounted cheek pouch. Letters and arrows indicate the fluorescence measured prior to (a), and 1 (b), 4 (c) and 30 min after (d) histamine application. Peak values of the two curves differed by ~10 min. A linear regression analysis after adjustment for this time shift of maximal response revealed a highly significant linear correlation between fluorescence of the 5-mm² area and FITC-dextran concentrations ($n = 12$, $r = 0.973$ and $p = 0.00001$).

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Fig. 3. a Changes in arteriolar diameter (means $\pm$ SEM and diameter at time 0 = 100%) measured using intravital microscopy in five groups of hamsters. Two groups were exposed to topical applications of SGH in 250 μ l normal saline for 9 min (SGH-0.5, $n = 6$, and SGH-1, $n = 5$). The fourth and fifth group were given topical applications of maxadilán (33 nM, $n = 5$, and 67 nM, $n = 7$) in 250 μ l saline, and the sixth group 250 μ l of normal saline in a similar way ($n = 6$). The four treated groups showed rapid and long-lasting dilation ($p < 0.05$) above the saline control group until 60 min. There was a significant difference ($p < 0.05$) between the 33- and the 67-nM maxadilán groups at 15 to 60 min after

stimulation. **b** Fluorescence (mean $\pm$ SEM) measured using intravital microscopy in five groups of hamsters with the same drug treatment and groups as in **a**. The four treated groups were significantly different ($p < 0.05$) from the control group 10–60 min after stimulation, and the larger dose of salivary homogenate (SGH-1) caused more plasma leakage than the smaller dose (SGH-0.5; * $p < 0.05$ vs. SGH-1). There was no difference between the SGH-0.5 group and the 33-nM maxadilán group ($p > 0.2$), but the effects induced by 67 nM maxadilán exceeded those induced by 33 nM (* $p < 0.05$ vs. 33 nM).

Topical application of 250 μ l of SGH-0.5 or the equivalent amount of SGH-1 on the HCP caused an immediate and long-lasting arteriolar dilation which was significantly different ($p < 0.05$) from the saline application control value at 2 min, reached its maximum after 10–15 min and remained above the initial control value until 60 min, when it returned to values before application. The dilation induced by SGH-1 was not significantly larger than that of SGH-0.5, although it remained above the values before application at 60 min (fig. 3a).

Maxadilán also caused immediate and long-lasting arteriolar dilation similar to that observed with SGH, which started to decrease 30 min after application of 33 nM maxadilán, while it remained 30% above the initial control value in the group receiving 67 nM maxadilán. Arteriolar dilation in the 67-nM maxadilán group was significantly greater ($p < 0.05$) than in the 33-nM maxadilán group from 15 min after challenge up to the final measurements (fig. 3a). The dilation curve induced by SGH-0.5 was almost identical to that produced by 33 nM maxadilán (fig. 3a).

Plasma Leakage Induced by SGH, Maxadilán and PACAP-38

Topical application of SGH-0.5 caused an increase in fluorescence which peaked after 20–25 min and plateaued until 60 min after its application, although 30 min after application arteriolar dilation had returned to values obtained before application. Consequently, the fluorescence which was still elevated after maximal dilation had been reached due to plasma extravasation and not vasodilation. The increase in fluorescence following SGH-1 was significantly larger (20%, $p < 0.05$) than that of SGH-0.5 (fig. 3b).

Both doses of maxadilán caused long-lasting increases in fluorescence that remained elevated after maximal dilation had been reached, thus showing that fluorescent plasma continued to leak from postcapillary venules during the entire experimental period (fig. 3b). Maxadilán (67 nM) caused 10–40% more plasma leakage than SGH-0.5 and SGH-1 at the same degree of dilation, but the effect of 33 nM of maxadilán was similar to that of SGH-0.5 or 10% below ($p > 0.2$ for differences between curve values). A second application of SGH-0.5 after 60 min caused

Fig. 4. a Changes in arteriolar diameter (means $\pm$ SEM; diameter at time 0 = 100%) assessed using intravital microscopy in hamsters exposed to topical applications of 50 nM PACAP-38. Maxadilane (67 nM)-induced effects were shown for comparison (fig. 3a); maxadilane caused a long-lasting dilation that remained above that induced by PACAP-38 50–60 min after its application (* $p <$

0.05). **b** Fluorescence (mean $\pm$ SEM) measured using intravital microscopy in hamsters exposed to topical applications of 50 nM PACAP-38. Maxadilane (67 nM)-induced effects (fig. 3b) are shown for comparison. There were no significant differences ($p > 0.05$) between the maxadilane ($n = 7$) and the PACAP-38 group ($n = 5$).

Fig. 5. a Arteriolar diameter changes (mean $\pm$ SEM) in 4 hamsters following application of 8 μ M M-65 and, after 30 min, M-65 and 67 nM maxadilane. There was only a short-lasting vasodilation after the first M-65 application, but not after the M-65-maxadilane combination. $n = 4$. **b** Fluorescence assessed using intravital mi-

croscopy in 4 hamsters showing no increase in plasma leakage after 8 μ M M-65 and inhibition of maxadilane-induced plasma leakage by 8 μ M M-65 applied prior to 67 nM maxadilane. The effect of maxadilane (fig. 3b, $n = 7$) was shown for comparison.

dilation of the same magnitude as the first application, but evoked no significant changes in plasma leakage (data not shown). PACAP-38 (50 nM) caused dilation and plasma leakage similar to that seen with maxadilane.

Effects of M-65 on SGH-, Maxadilane- and PACAP-38-Induced Dilation and Plasma Leakage

M-65 is a maxadilane-derived mutant peptide (deletion of amino acids 25–41 in the maxadilane molecule)

Fig. 6. a Mean arterial diameter changes (% of diameter at time 0) in 5 hamsters following application of 8 μ M M-65 + SGH, with a temporary inhibition of SGH-induced vasodilation by M-65. The vasodilation induced by SGH without M-65 was of the same magnitude as that for SGH-1 (fig. 3a). **b** Fluorescence measured

using intravital microscopy in 5 hamsters revealed no consistent increase in plasma leakage after combined application of M-65 + SGH-1, but an increase following SGH-1 30 min after M-65, thus a temporary inhibition of SGH-induced plasma leakage. The effect of SGH-0.5 (fig. 3b) was shown for comparison.

which has been shown to bind to PAC-1 receptors generating a maxadilan antagonistic effect [16, 21]. When an 8- μ M solution of M-65 was applied to the cheek pouch, there was a short-lasting dilation (fig. 5a), but there was no increase in plasma leakage. Furthermore, when M-65 was given prior to maxadilan, it totally blocked its vasodilator and plasma leakage effects (fig. 5b). M-65 also inhibited the vasodilation and plasma leakage caused by SGH (fig. 6). The inhibition resolved after a 30-min washout period, and the response to a second dose of SGH without M-65 was of the same magnitude as that induced by SGH (fig. 3b). In three experiments, 8 μ M M-65 was given prior to PACAP-38 application and there was no difference compared with the effects of PACAP-38 (fig. 7).

Effects of PACAP6-38 on Maxadilan-Induced Dilation and Plasma Leakage

PACAP6-38 is a competitive antagonist to PACAP-38, missing a sequence of 6 amino acids. Topical application of 4 μ M PACAP6-38 prior to 67 nM maxadilan resulted in no arteriolar dilation (fig. 8a) and an inhibition of the plasma leakage effect of maxadilan 35 min after its application (fig. 8b). The inhibition was not complete, which was confirmed by measurements of dilation suggesting that PACAP6-38 has a slight agonistic effect on plasma leakage. Application of 0.4 and 4 μ M PACAP6-38 resulted in similar arteriolar dilation changes as seen after

4 μ M PACAP6-38 + maxadilan. There was no plasma leakage induced by 400 nM PACAP6-38, but the effect of 4 μ M was similar to that exerted by PACAP6-38 + maxadilan.

Effects of HOE-140, Mepyramine and L-NA on SGH- and Maxadilan-Induced Dilation and Plasma Leakage

Mean basal arteriolar diameter was 47.8 ± 4.1 μ m in the saline control group, 39.9 ± 6.4 μ m in the SGH-0.5 group, 46.2 ± 4.5 μ m in the SGH-1 group, 46.5 ± 3.9 μ m in the HOE-140 group, 38.9 ± 7.5 μ m in the mepyramine group and 44.4 ± 2.2 μ m in the L-NA group. There were no significant differences in basal diameters in these groups. Treatment of the HCP with HOE-140, mepyramine or L-NA prior to application of SGH-0.5 had no effect on arteriolar dilation, but resulted in a 20 % reduction in plasma leakage ($p < 0.05$) in the HOE-140-treated group (fig. 9). A second application, of SGH-0.5 induced dilation similar to the first application, but no further increase could be observed in plasma leakage (data not shown). Similar to SGH-0.5 experiments, L-NA applied prior to maxadilan did not inhibit maxadilan-induced effects (data not shown). The possible inhibition of the maxadilan-induced dilation and plasma leakage by the bradykinin antagonist HOE-140 was tested in 3 hamsters: 1 treated with maxadilan (positive control; fig. 3) and 2 with 0.5 μ M HOE-140 during the 5 min prior to

Fig. 7. a Mean arteriolar diameter changes (% of diameter at time 0) in 3 hamsters following application of 8 μM M-65 + 50 nM PACAP-38, with no difference compared with the effect of PACAP-38. The results of 5 hamsters given 50 nM PACAP-38 (fig. 4a) were shown for comparison. **b** Using fluorescence mea-

surements in 3 hamsters, plasma leakage did not differ after application of 8 μM M-65 + 50 nM PACAP-38 and PACAP-38. The results of 5 hamsters given 50 nM PACAP-38 (fig. 4b) were shown for comparison.

Fig. 8. a Arteriolar diameter changes (% of diameter at time 0; mean $\pm$ SEM) in 4 hamsters following application of 4 μM PACAP6-38 + 67 nM maxadilan, showing significant inhibition of maxadilan-induced dilation (* $p < 0.05$). Results in hamsters given 4 ($n = 3$) or 0.4 μM PACAP6-38 ($n = 1$) were not different from those treated with PACAP6-38 + maxadilan. The results in 7 hamsters given 67 nM maxadilan (fig. 3a) were shown for com-

parison. **b** Fluorescence measured in 4 hamsters showing a temporary increase in plasma leakage after application of 4 μM PACAP6-38 + 67 nM maxadilan followed by a significant inhibition of the maxadilan-induced plasma leakage (* $p < 0.05$) 35–60 min after application. A similar increase in plasma leakage was seen with 4 μM PACAP6-38, but there was no increase after application of 0.4 μM PACAP6-38 (means $\pm$ SEM).

and 30 min after maxadilan application. There were neither differences in the vasodilatory responses nor in the plasma leakage response between these 3 hamsters (data not shown).

Discussion

FITC-dextran has been well established as a plasma marker to measure vascular permeability increase in postcapillary venules [33, 42]. Several studies using FITC-

Fig. 9. a Changes in arteriolar diameter (means $\pm$ SEM; diameter at time 0 = 100%) measured using intravital microscopy in four groups with 4–6 hamsters each exposed to topical applications of SGH in 250 μ l normal saline (SGH-0.5). Prior to application of SGH-0.5, the prepared cheek pouches were locally treated with HOE-140 (0.5 μ M), mepyramine (25 μ M) or L-NA (10 μ M) for 5 min without interruption of superfusion. SGH-0.5-induced dilation (fig. 3a) was shown for comparison. Dilation did not sig-

nificantly differ in the four groups. **b** Fluorescence (means $\pm$ SEM) measured using intravital microscopy in four groups with 4–6 hamsters each exposed to topical applications of drugs (see fig. 9a). SGH-0.5-induced increases in plasma leakage (fig. 3b) were shown for comparison. Increases in fluorescence did not differ in the four treatment groups until 45 min after SGH application, when the HOE-140 group showed a 20% reduced plasma leakage (* $p < 0.05$) compared with the SGH-0.5 group.

dextran to measure microvascular permeability increase or plasma leakage have shown that the number of leaks counted microscopically was correlated with the clearance of FITC-dextran from the cheek pouch measured in the superfusion fluid [35–39]. A linear correlation between fluorescence intensity on a TV monitor and FITC-dextran vascular and tissue concentrations in the HCP has also been found [40]. In the present study, a digital camera and computer software (AxioVision) were used to measure fluorescence in a rectangular area of 5 mm². In order to validate this refinement of the original method of measuring plasma leakage in the microcirculation, FITC-dextran concentrations in the superfusion solution were determined concomitantly with digital camera fluorescence recordings before and after histamine application. These experiments confirmed that the dynamics of the recorded fluorescence corresponded with FITC-dextran concentrations, as a linear correlation was obtained between fluorescence and the concentration of FITC-dextran in the superfusion fluid. Therefore, our results demonstrate that the fluorescence measurements in the restricted area were representative of the vascular permeability changes in the entire cheek pouch preparation.

The vasodilatory effects of salivary gland homogenates and their capacity to enhance *Leishmania* infectivity have already been recognized [5, 12]. Apart from the

vasodilatory peptide maxadilan, a broad range of proteins has been identified in the salivary gland of *L. longipalpis* [26], possibly indicating that although maxadilan might be the major vasodilator of saliva, other proteins may also contribute to the pro-inflammatory effects of *L. longipalpis* saliva. As a first approach, the immediate inflammatory changes in SGH isolated from the sand fly *L. longipalpis* were compared with those induced by recombinant maxadilan immediately after application to the HCP microcirculation, a skin-like tissue. However, it remains to be determined how the parasites benefit from these pro-inflammatory or vasoactive effects at the moment of infection. The enhancement could be due to arteriolar dilation, but it could also be due to other vascular effects related to the activation of endothelial cells in postcapillary venules, possibly via activation of adhering neutrophils. The postcapillary venules are known to respond to inflammatory mediators and cytokines, some of which may be induced by maxadilan itself [43].

The effect of maxadilan lasted longer than that of PACAP-38 and was inhibited by the maxadilan mutant M-65, which had no influence on the PACAP-38 effect. It is possible that the maxadilan peptide is more selective in stimulating PAC-1 receptors, while PACAP-38 may also stimulate VPAC1 and VPAC2 receptors [44].

Although PACAP6-38 acts as a PACAP receptor antagonist in most cells/tissues, agonistic functions in other biological systems have also been reported [45]. In our model of skin microcirculation, the PACAP6-38-induced vasodilation differed from that induced by maxadilan or PACAP-38, and, furthermore, counteracted maxadilan-induced vasodilation. Using plasma leakage as determining factor, however, PACAP6-38 induced similar responses, albeit at 60-fold higher molar concentrations than maxadilan.

It has been observed that PACAP could act as an activator of human neutrophils and monocytes [30, 31], and thus it is possible that the hamster neutrophils may be activated by PACAP-38 or maxadilan, hence releasing granule proteins which eventually cause endothelial cell separation and subsequent long-lasting plasma leakage.

It is well established that a non-inflammatory physiological vasodilator such as acetylcholine may produce increases in blood flow and small vein pressure comparable to that induced by bradykinin and histamine, but without provoking plasma extravasation [46, 47]. Actually, other types of vasodilators, e.g. β_2 -adrenoceptor agonists (e.g. terbutaline and salbutamol) and phosphodiesterase inhibitors (e.g. theophylline, milrinone and rolipram) may counteract histamine- and bradykinin-induced plasma leakage [23, 47, 48]. Thus, acetylcholine causes vasodilation without plasma leakage, but histamine and bradykinin cause arteriolar dilation and also act on the postcapillary venular endothelium, hence inducing plasma extravasation. The vasodilation induced by exogenous bradykinin and histamine is of brief duration, and the concomitant increase in plasma leakage is resolved within 30 min, consequently markedly different from effects induced by SGH, maxadilan and PACAP-38. These three components caused a progressive and long-lasting vasodilation which peaked after 10–15 min, yet the effect was coupled to an independent increase in plasma leakage. Of note, the extravasation of FITC-dextran was protracted in the SGH and maxadilan groups, but started to decrease 30 min after application of PACAP-38. The long-lasting vasodilation and persistent increase in plasma leakage by maxadilan may be explained by studies investigating maxadilan-induced stimulation of macrophages and dendritic cells resulting in pro-inflammatory cytokine release [43, 49].

In 2001, in a study by Morris et al. [41] in mice, half a salivary gland produced effects similar to 3 ng of maxadilan. In our study, SGH-0.5 produced effects equal to 60 ng of maxadilan (250 μ l of a 33-nM solution = 60 ng) in terms of vasodilation and plasma leakage. This difference

could be due to differences in the content of maxadilan depending on the source *L. longipalpis* [50].

The possibility that sand fly saliva and maxadilan might activate the kinin system was explored using the bradykinin antagonist HOE-140. Although HOE-140 reduced plasma leakage by 20%, this effect was small compared with the inhibition seen with the maxadilan mutant M-65, which almost completely inhibited the plasma leakage induced either by SGH or maxadilan. Treatment of the HCP with HOE-140 or L-NA prior to maxadilan resulted in no visible changes, suggesting that kinin formation does not play an important role in the microvascular effects induced by maxadilan (data not shown). Given indications that *L. chagasi* promastigotes can induce edema and infect macrophages through the activation of the kinin pathway [34], it will be interesting to determine if the convergence of these responses may have short- or long-lasting effects on the host-parasite relationship. This hypothesis merits analysis in light of recent studies showing that bradykinin, recently characterized as a potent maturation signal for dendritic cells, links innate to type-1 adaptive immunity in mice infected with *Trypanosoma cruzi* [51] (for a review, see Scharfstein et al. [52]).

Although limited to studies with SGH and maxadilan so far, our results suggest that sand fly saliva may simultaneously induce vasodilation and plasma leakage, the effects being most probably exerted via the PACAP receptor PAC1. In 1997, Moro and Lerner [15] revealed that maxadilan is a PAC1 receptor agonist without structural similarity to PACAP. Warren et al. [22, 23] reported that several vasodilators, e.g. PACAP, enhanced the plasma leakage effect of bradykinin, and in 1997 Cardell et al. [24] showed that PACAP-38 stimulation of rat skin caused plasma leakage and suggested that it could be due to histamine release. More recent studies showed that in the human nose PACAP-38 increased albumin concentration in nasal secretion fluid, which is a sign of plasma leakage [25]. The selective inhibitor used in our study, a 44-amino acid mutant of the 61-amino acid peptide maxadilan, had minor inflammatory effects (plasma leakage) when applied for 10 min at concentrations 100 times exceeding that of maxadilan. Notably, the M-65 mutant protein completely inhibited both vasodilation and plasma leakage induced by maxadilan. PACAP-38 applied to the cheek pouch in the same way as maxadilan caused vasodilation and plasma leakage corresponding to those of maxadilan, suggesting that part of the immediate inflammatory effect of PACAP-38 could be exerted via PAC1 receptor stimulation.

Conclusion

Apart from their predicted vasodilatory effects, SGH, maxadilan and PACAP-38 were able to independently evoke plasma leakage in the cheek pouch. The finding that the M-65 mutant of maxadilan reduced both vasodilation and plasma leakage induced by SGH or maxadilan suggests that their vasoactive and pro-inflammatory effects are at least partially mediated by activation of PAC1 receptors. Vascular permeability increase as well as the well-recognized vasodilation induced by SGH and its component maxadilan might also be an important factor

for the development of inflammation at early stages of *Leishmania* infection. Further studies are necessary to elucidate to what extent the induced plasma leakage is due to consequences of neutrophil activation.

Acknowledgments

Our study was supported by grants from CNPq (National Council for Scientific and Technological Development) project no. 410552/2006-2 and FAPERJ (Foundation for Research Support of the Rio de Janeiro State) E-26/100.938/2007.

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