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Highlights

- Maxadilan diminishes mustard oil-induced neurogenic edema in the mouse ear.
- Maxadilan decreases neurogenic vasodilation and plasma leakage in the skin.
- Maxadilan reduces mustard oil-evoked substance P release in the mouse ear.
- VIP decreases neurogenic vasodilation, but not plasma leakage and edema formation.
- Neither maxadilan, nor VIP influences mustard oil-induced neutrophil accumulation.

http://dx.doi.org/10.1016/j.neuropharm.2014.06.019
0028-3908/

Neuropharmacology xxx (2014) 1–10

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The selective PAC1 receptor agonist maxadilan inhibits neurogenic vasodilation and edema formation in the mouse skin

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ARTICLE INFO

Article history:
Received 16 April 2014
Received in revised form
14 June 2014
Accepted 16 June 2014
Available online xxx

Keywords:
PACAP
Maxadilan
VIP
Neurogenic inflammation
Plasma leakage
Vasodilation

ABSTRACT

We have earlier shown that PACAP-38 decreases neurogenic inflammation. However, there were no data on its receptorial mechanism and the involvement of its PAC1 and VPAC1/2 receptors (PAC1R, VPAC1/2R) in this inhibitory effect.

Neurogenic inflammation in the mouse ear was induced by topical application of the Transient Receptor Potential Ankyrin 1 (TRPA1) receptor activator mustard oil (MO). Consequent neurogenic edema, vasodilation and plasma leakage were assessed by measuring ear thickness with engineer's micrometer, detecting tissue perfusion by laser Doppler scanning and Evans blue or indocyanine green extravasation by intravital videomicroscopy or fluorescence imaging, respectively. Myeloperoxidase activity, an indicator of neutrophil infiltration, was measured from the ear homogenates with spectrophotometry. The selective PAC1R agonist maxadilan, the VPAC1/2R agonist vasoactive intestinal polypeptide (VIP) or the vehicle were administered i.p. 15 min before MO. Substance P (SP) concentration of the ear was assessed by radioimmunoassay.

Maxadilan significantly diminished MO-induced neurogenic edema, increase of vascular permeability and vasodilation. These inhibitory effects of maxadilan may be partially due to the decreased substance P (SP) levels. In contrast, inhibitory effect of VIP on ear swelling was moderate, without any effect on MO-induced plasma leakage or SP release, however, activation of VPAC1/2R inhibited the increased microcirculation caused by the early arteriolar vasodilation. Neither the PAC1R, nor the VPAC1/2R agonist influenced the MO-evoked increase in tissue myeloperoxidase activity.

These results clearly show that PAC1R activation inhibits acute neurogenic arterial vasodilation and plasma protein leakage from the venules, while VPAC1/2R stimulation is only involved in the attenuation of vasodilation.

1. Introduction

Transient Receptor Potential Ankyrin 1 (TRPA1) is known to mediate pain and inflammatory processes, but its involvement in cold- and somatosensation is still debated (Bautista et al., 2006; Story et al., 2003). These non-selective cation channels are expressed on peripheral and central terminals of capsaicin-sensitive peptidergic primary afferent neurons, where they signal and amplify nociceptive stimuli. Several agents have been shown to activate TRPA1 receptors, including mustard oil (MO, also known as allyl isothiocyanate, AITC), formalin, thio-sulfinate in garlic, α,β-unsaturated aldehydes in cinnamon, air pollutants, nicotine, tear gas components, reactive oxygen species and chlorine (Hinman

Abbrevations: AITC, allyl isothiocyanate; ANOVA, repeated measures analysis of variance; CGRP, calcitonin gene-related peptide; COPD, chronic obstructive pulmonary disease; ICG, indocyanine green; IL, interleukin; i.p., intraperitoneal; i.v., intravenous; MO, mustard oil; MPO, myeloperoxidase; OD, optical density; PACAP, pituitary adenylate cyclase activator polypeptide; PO, paraffin oil; s.c., subcutaneous; SEM, standard error of mean; SP, substance P; TNFα, tumor necrosis factor α; TRPA1, transient receptor potential ankyrin 1; VIP, vasoactive intestinal polypeptide.

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http://dx.doi.org/10.1016/j.neuropharm.2014.06.019
0028-3908/

et al., 2006). The natural plant-derived irritant, MO, stimulates TRPA1 on sensory nerve endings through covalent modification of cysteines on the intracellular C-terminal domain of the channel (Bautista et al., 2006; Hinman et al., 2006; Macpherson et al., 2007; McNamara et al., 2007). Sensory neuropeptides, such as calcitonin gene-related peptide (CGRP) and tachykinins (substance P: SP and neurokinins A and B), are released from these stimulated nerve terminals that induce a rapid inflammatory response (arteriolar vasodilation, plasma extravasation, recruitment of leukocytes and mast cell degranulation) locally in the innervated area (Szolcsányi, 1988). Moreover, sensory nerve endings also release neuropeptides, like somatostatin and pituitary adenylate-cyclase activating polypeptide (PACAP), exerting anti-inflammatory actions. Neurogenic inflammation plays a key pathogenetic role in a variety of different acute and chronic inflammatory diseases (Chiu et al., 2012). This is a basically different inflammatory mechanism compared to immune cell-mediated processes, it is often the very early initiation step even in chronic diseases including allergic contact dermatitis, atopic dermatitis, rosacea, migraine, allergic rhinitis, sarcoidosis, rheumatoid arthritis, psoriasis, asthma and COPD (Abad et al., 2006; Anichini et al., 1997; Aubdool and Brain, 2011; Bánvölgyi et al., 2005; Geppetti et al., 2005; O'Connor et al., 2004; Pisi et al., 2009; Raychaudhuri and Raychaudhuri, 2004; Teresiak-Mikołajczak et al., 2013). This triggers and remarkably augments further cellular pathways. Sensory nerve terminal activation and the neurogenic inflammatory component is basically not inhibited by the conventional anti-inflammatory drugs (cyclooxygenase inhibitors), but non-steroidal anti-inflammatory drugs might have an inhibitory action in case of nerve terminal sensitization by prostaglandins in the inflamed tissue (Pethő and Reeh, 2012). Furthermore, glucocorticoids are only moderately effective in extremely high doses in which they exert many severe side-effects that limit their clinical applications (reviewed in: Helyes et al., 2003). Therefore, there is an urgent need to develop a potential candidate which interacts with this crucial pathophysiological mechanism.

Several neuropeptides are known to be involved in the regulation of the neuro-immuno-endocrine system (Ganea and Delgado, 2002). Among these, PACAP is a pleiotropic and multifunctional neuropeptide, which is widely distributed in the brain, peripheral nervous system, cardiovascular, gastrointestinal and respiratory tracts. Besides its diverse effects in these organs, its inhibitory action on cellular and vascular components of inflammation and its vasodilatory actions have also been investigated in numerous studies (reviewed in: Vaudry et al., 2009). Anti-inflammatory properties of the peptide lead to its significant ameliorative effect in animal models of septic shock, stroke, diabetic nephropathy and colitis (Azuma et al., 2008; Banki et al., 2013; Dejda et al., 2011; Martinez et al., 2002). The effects of PACAP are mediated by G protein-coupled receptors: the specific PAC1 receptor with 8 different splice variants, and VPAC1 and VPAC2 receptors which bind PACAP and VIP with the same affinity (reviewed in: Vaudry et al., 2009). PAC1R is mainly expressed on smooth muscle cells, neurons, endothelial cells and peritoneal macrophages. VPAC1 receptor is constitutively expressed in the dorsal horn of the spinal cord, on T-lymphocytes, macrophages, monocytes, mast cells and dendritic cells, while the expression of VPAC2 is inducible on these cells (Delgado et al., 1996, 1999; Delgado and Ganea, 2013; Ganea, 1996; Vaudry et al., 2009).

Similarly to PACAP, the VPAC1/2 agonist VIP and the selective PAC1 agonist maxadilan have also been reported to exert anti-inflammatory and vasodilatory actions. Alterations in the level of the 28 amino acid neuropeptide VIP were shown in several immunological diseases, like sepsis, rheumatoid arthritis, lupus, autoimmune thyroiditis, while its involvement in neurogenic inflammatory disorders has also emerged (Delgado and Ganea, 2013; Lundy and Linden, 2004; Teresiak-Mikołajczak et al., 2013; Wu et al., 2011). We learned from studies with VIP-deficient mice that endogenous VIP exerts anti-inflammatory properties in LPS-induced septic shock, asthma and pulmonary hypertension (Delgado and Ganea, 2013; Hamidi et al., 2006).

The specific PAC1 receptor agonist maxadilan is a vasoactive compound, which was originally isolated from the salivary gland extract of *Lutzomyia longipalpis*, the vector of leishmaniasis (Lerner et al., 1991; Moro and Lerner, 1996). The peptide was named after its potent vasodilating effect, which was found to be endothelium-independent, and was even shown in the human skin by laser Doppler method (Grevelink et al., 1995; Lerner et al., 1991). Vasoactive properties of maxadilan include increase in blood flow, inhibition of platelet aggregation and blood coagulation. Its receptor-binding affinity is high, resulting in prolonged vasoactive effects persisting for 2 days (Grevelink et al., 1995). Maxadilan was also reported to exhibit profound anti-inflammatory properties (Bozza et al., 1998; Qureshi et al., 1996; Soares et al., 1998).

Besides the pivotal role of PACAP in non-neurogenic inflammation, involvement of PACAP in neurogenic inflammation was also investigated. Among several other neuropeptides, PACAP is also released from sensory nerve terminals and exhibits anti-inflammatory properties by inhibiting the stimulated release of neuropeptides including CGRP, SP and somatostatin (Fahrenkrug and Hannibal, 1998; Németh et al., 2006). Németh et al. (2006) reported that mustard oil-induced neurogenic edema and albumin extravasation were diminished by systemic PACAP treatment. However, no studies have been performed to elucidate the contribution of its three receptors to the vascular inflammatory reactions of the neuropeptide. The aim of the present study was to examine the involvement of PAC1 and VPAC1/2 receptors in the anti-inflammatory potential of PACAP in neurogenic inflammation.

2. Materials and methods

2.1. Animals

Experiments were performed using 3-month-old male and female CD1 mice, since we have never found gender difference in this model in earlier experiments (Pozsgai et al., 2010, 2012). Mice were kept in the Laboratory Animal House of the Department of Pharmacology and Pharmacotherapy of the University of Pécs at 24–25 °C and provided standard mouse chow and water *ad libitum*. All experimental procedures were carried out in accordance with approved protocols (University of Pécs; BA02/2000-15024/2011). All efforts were made to minimize animal suffering and to reduce the number of animals used. The applied solutions were freshly prepared before each experiment.

2.2. Measurement of mustard oil- and formalin-induced neurogenic edema formation in the mouse ear

Mice were treated either with PAC1 receptor agonist maxadilan (100 μg kg^{-1}) or VPAC1/2 agonist VIP (100 μg kg^{-1}) or saline (10 ml kg^{-1}) intraperitoneally (i.p.) 15 min prior to the experiments. Dose of the applied agonists was determined on the basis of earlier experiments with the PAC1 and VPAC1/2 receptor agonist PACAP-38 in the same or similar models (Németh et al., 2006; Helyes et al., 2007), as well as potencies of these peptides are similar to PACAP-38 on isolated primary sensory neurones and nerve terminals (unpublished data). Mice were anesthetised with ketamine and xylazine (100 mg kg^{-1} and 5 mg kg^{-1}, i.p., respectively) before the experiment, and were kept under anesthesia by injecting 1/2–1/3 of the applied initial dose every hour. Either 10 μl of 1 or 5% mustard oil dissolved in paraffin oil (PO) ($n = 4$–5 and 4–6 mice in each experimental group, respectively) or 10 μl of 5% formalin dissolved in distilled water ($n = 4$–5 mice/group) was applied topically on both surfaces of the ear at the beginning of the experiment and 1 h later. Ear thickness was measured with engineer's micrometer (Moore and Wright, Sheffield, UK) with an accuracy of 0.1 mm before the treatment as control and 30 min after the application of mustard oil or formalin, and later every hour until the end of the 6-h period. Data are shown as means ± SEM of percentage increase of ear thickness compared to the initial controls.

2.3. Measurement of Evans blue-bound albumin extravasation in the mouse ear

Intraperitoneal treatment of the mice was performed as described above (Section 2.2.). Mice ($n = 4$–5/group) were anesthetised with urethane (1.2 g kg^{-1}) and their core body temperature was maintained at 38 °C with a heating pad. Evans blue (25 mg kg^{-1})

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was injected intravenously (i.v.) at least 10 min prior to experiment to allow distribution of the dye. Evans blue is a tetrasodium diazo salt, which binds plasma albumin with high affinity. Therefore, Evans blue is a suitable dye for the detection of plasma leakage when injected into the bloodstream. Increasing blue color outside the vessels indicated enhanced vascular permeability. Plasma leakage was detected by Nikon intravital videomicroscope with 1× objective and 2× optical zoom. 3 images were taken as a control before the topical application of 20 μl paraffin or 5% mustard oil. Pictures were taken in every 30 s for 30 min after paraffin or mustard oil treatment. Evans blue accumulation was assessed by image analysis performed with Image-Pro Plus 7.0.0.591 (Media Cybernetics Inc., MD, USA) software by measuring the blue optical density of each image. The small white shining areas due to the mustard oil smearing were excluded by setting the threshold of blue component of the RGB scale to 182. Data were expressed in % change, as mean ± SEM of the control values.

2.4. Fluorescence imaging of vascular leakage in the mouse ear

Intravenously injected indocyanine green (ICG), a fluorescent cyanine dye, binds to plasma proteins and remains in the healthy vasculature. However, under inflammatory conditions, it can be used to evaluate inflammatory hypervascularisation and capillary leakage. ICG (0.5 mg kg^{-1}) was dissolved freshly in 5 w/v% aqueous solution of Kolliphor HS 15 and a macrogol-based surfactant (Kirchherr et al., 2009), and injected intravenously under ketamine – xylazine anesthesia (100 mg kg^{-1} and 5 mg kg^{-1}, i.p., respectively) immediately before the 5% mustard oil smearing. Intraperitoneal treatment of the mice ($n = 4–5$/group) was performed as described above (Section 2.2.). Animals were imaged 5, 10, 20 and 30 min after the topical application of 20 μl 5% mustard oil with the IVIS Lumina II optical imager (Perkin–Elmer, Waltham, MA, USA). Two regions of interest (ROIs) were chosen representing both ears of the mice. Imaging was performed with the following parameters: auto acquisition time, F/stop = 1, Binning = 2, excitation: 745 nm, emission filter: ICG-specific (>800 nm), long-bandpass. Data were analyzed with Living Image® software. A calibrated unit of the fluorescence, the radiant efficiency ([photons/s/cm2/sr]/[μW/cm2]) originating from the ROIs was used for further analysis.

2.5. Determination of cutaneous blood perfusion in the mouse ear

Intraperitoneal treatment of the mice was performed as described above (Section 2.2.). Mice ($n = 7$/group) were anesthetised with ketamine and xylazine (100 mg kg^{-1} and 5 mg kg^{-1}, subcutaneously (s.c.), respectively) and their body temperature was maintained with a heating pad. Cutaneous blood flow was recorded by laser Doppler perfusion imaging (Periscan PIM-II, Perimed, Sweden). Experiments were carried out as previously described by Pozsgai et al. (2012). Briefly, scanned area was set to 30 × 64 sampling points, and imaging of the head and both ears was performed in every 2 min. Three images were taken as control at the beginning of the experiment. After determination of the baseline, 20 μl of 5% mustard oil was applied topically to the dorsal surface of the right ear, while the left ear was treated with the solvent, paraffin oil (PO). Mustard oil-induced neurogenic inflammation is accompanied by enhanced blood flow. Blood perfusion of the ears was detected for 30 min after the induction of neurogenic inflammation. A color code was used to visualize blood flow values: black and dark blue represented areas with low blood perfusion, while light blue, green and yellow to red indicated increasing blood flow values. Two regions of interest (ROIs) were chosen representing the total area of both ears. Blood flow of the ears was calculated by comparing mean microcirculation values of the ROIs to those of measured on the three baseline images. In order to exclude systemic perfusion changes, blood flow values of the vehicle-treated ears were subtracted from those of the ones treated with 5% mustard oil.

2.6. Measurement of myeloperoxidase (MPO) activity in the mouse ear

6 h after the first topical application of 5% AITC, ears were removed, frozen in liquid nitrogen and stored at −80 °C until further processing. There were 4–6 animals involved in each experimental group. Measurement of neutrophil accumulation was performed as described previously (Bánvölgyi et al., 2004). Briefly, ears were thawed and homogenized in 2 ml 20 mM potassium-phosphate buffer (pH 7.2–7.4) and centrifuged at 10,000 g, 4 °C for 10 min. The pellet was resuspended in 4 ml 50 mM potassium-phosphate buffer containing 0.5% hexadecyltrimethylammonium (HTAB) (pH 6.0) and centrifuged again. MPO activity was assayed from the supernatant using H_2O_2-3,3′,5,5′-tetramethylbenzidine (TMB/H_2O_2). Neutrophil accumulation in the ear samples was assessed by comparing MPO enzyme activity of the samples to a human standard. The optical density (OD) was measured twice with 5 min difference at 620 nm using a microplate reader (Labsystems) and plotted. The reaction rate (ΔOD/time) was derived from an initial slope of the curve. A calibration curve was then produced, with the rate of reaction plotted against the standard samples. Data were expressed in U/g wet tissue.

2.7. Measurement of SP concentration of the mouse ear by radioimmunoassay (RIA)

Ears were removed 20 min after the mustard oil stimulation and frozen at −20 °C until further processing. Homogenization was performed as described for the MPO measurement, but peptide concentration was determined from 30 μl of the supernatant after the first centrifugation (described in Section 2.6.). Radioimmunoassay method was developed in our laboratory as described in details elsewhere

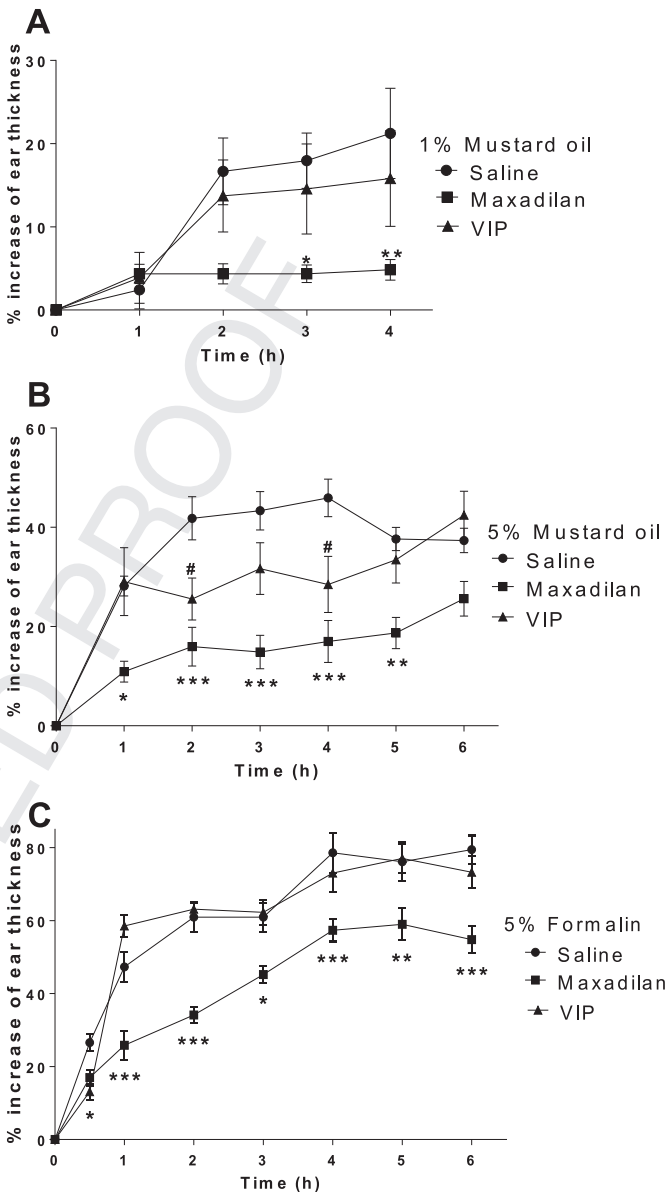

Fig. 1. Effect of i.p. maxadilan and VIP on 1% mustard oil- (A), 5% mustard oil- (B) and 5% formalin-induced (C) swelling in the mouse ear. Two-way ANOVA followed by Bonferroni's test; $n = 6–12$ per group; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ maxadilan vs. saline; $^{\#}p < 0.05$ VIP vs. saline.

and has proved to be specific, sensitive and valid for the measurement of sensory neuropeptide concentration (Helyes et al., 1997, 2007; Németh et al., 1996, 1998, 1999, 2006). Detection limits of the assays were 2 fmol/tube. Synthetic peptides were used as RIA standards ranging from 0 to 1000 fmol/ml. The assay was prepared in 1 ml 0.05 mol/l (pH 7.4) phosphate buffer containing 0.1 mol/l sodium chloride, 0.25 w/v% BSA and 0.05 w/v% sodium azide. The antiserum (100 μl, 1:310,000 dilution), the RIA tracer (100 μl 5000 cpm/tube), the standard or unknown samples (30 μl) and the assay buffer were added to polypropylene tubes. After 72 h of incubation at 4 °C, the antibody-bound peptide was separated from the free peptide with 100 μl separating solution (10 g charcoal, 1 g dextran and 0.5 g commercial fat-free milk powder in 100 ml distilled water). Radioactivity of the precipitates was assessed by a gamma counter (Gamma, type: NZ310) after a 15 min centrifugation at 3000 r.p.m. Peptide concentration of the unknown samples was read from a calibration curve. Peptide concentration in fmol per ear was expressed.

2.8. Statistical analysis

Statistical analysis was performed by GraphPad software. One-way or Two-way repeated measures analysis of variance (ANOVA) with Bonferroni correction was used to detect significant differences between groups in all experiments. *P* value less than 0.05 was considered to be statistically significant.

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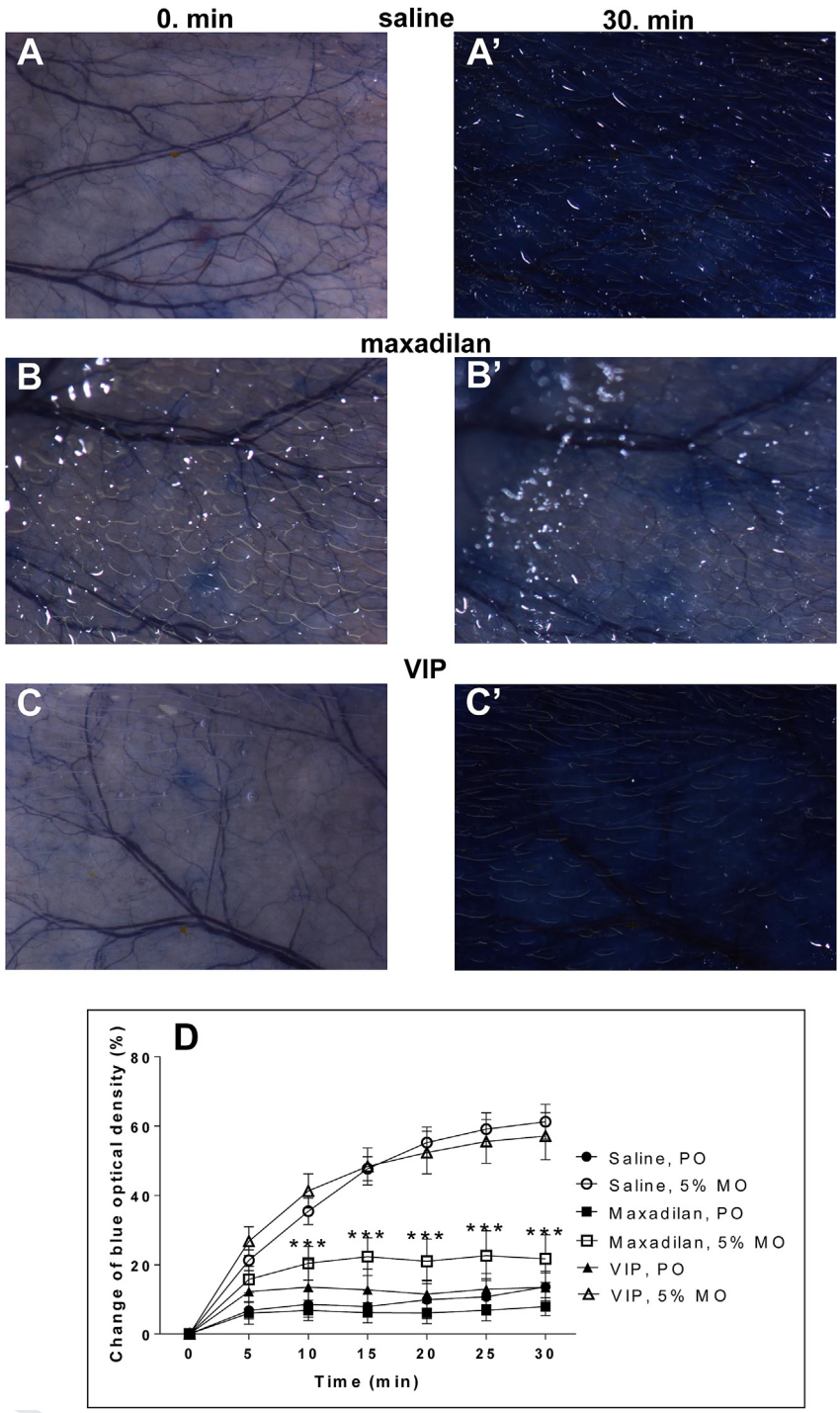

Fig. 2. Representative intravital videomicroscopic images of 5% mustard oil-treated ears of saline- (A, A'), maxadilan- (B, B') and VIP-treated (C, C') mice taken at the time of mustard oil treatment (A, B, C) and 30 min later (A', B', C'). Increasing blue color in the extravasal compartment indicates enhanced leakage of Evans blue-bound albumin due to increased permeability of the postcapillary venules. Effect of VIP and maxadilan on 5% mustard oil (MO)-induced plasma leakage in the ear (D). Two-way ANOVA followed by Bonferroni's test; $n = 4–5$ per group; $^{***}p < 0.001$ maxadilan, 5% mustard oil (MO) vs. saline, 5% MO and VIP, 5% MO. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.9. Drugs and chemicals

Evans blue, human MPO standard preparation and H_2O_2-3,3′,5,5′-tetramethylbenzidine (HTMB) were obtained from Sigma (St Louis, MO, USA), while maxadilan from Bachem (Switzerland). Ketamine was purchased from Richter Gedeon (Hungary) and xylazine from Eurovet Animal Health BV (Netherlands). Formalin (Formaldehydum solutum 37%; Ph.Hg. VII.) and paraffin were obtained from the Pharmacy of the University of Pécs and urethane was ordered from Spektrum 3D (Hungary). The tracer for RIA measurements (mono-^{125}I-SP) was purchased from Perkin–Elmer (Boston, USA).

3. Results

3.1. Effect of VIP and maxadilan on neurogenic edema formation in the mouse ear

Mustard oil and formalin activate capsaicin-sensitive sensory nerve terminals via TRPA1 receptor causing neurogenic edema (Hinman et al., 2006; Bessac et al., 2009; Wei et al., 2010; Kunkler et al.,

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2011). Maximal increase of ear thickness was ~20%, ~45% and ~80% after topical application of 1 and 5% mustard oil and 5% formalin, respectively.

Topical application of 1% mustard oil led to a maximum of 21.2% increase in ear thickness after 4 h in the control, saline-treated animals. The mustard oil-induced edema was absent in the maxadilan-treated group: maxadilan significantly counteracted the neurogenic edema formation both 3 and 4 h after the topical application of 1% MO with a maximum of 4.8% increase in ear thickness. VIP treatment did not lead to significant inhibition of the neurogenic edema (Fig. 1A).

5% MO caused markedly greater edema compared to the 1% solution with a maximum of 45.9% increase in the saline-treated animals. Maxadilan significantly decreased this neurogenic edema formation in the first 5 h of the experiment, and in case of this greater swelling VIP also induced significant inhibition after 2 and 4 h. However, similarly to the results obtained with 1% MO, the inhibitory effect of maxadilan was significantly greater than that of VIP throughout the whole experiment, the maximal increase was only 25.6% (Fig. 1B).

Neurogenic edema in response to 5% formalin was also determined. No difference was observed between the vehicle- and the VIP-treated groups; maximal increase of ear thickness was 79.4% and 77%, respectively. Maxadilan significantly counteracted the formalin-induced neurogenic edema during the 6 h of experiment (Fig. 1C).

Based on these results, 5% MO was found to be the most suitable compound to examine the effect of maxadilan and VIP on neurogenic inflammation. Therefore, further experiments were performed using 5% MO.

3.2. Effect of VIP and maxadilan on plasma extravasation in the mouse ear

As shown in Fig. 2, 5% MO increased the plasma leakage indicated by the excessive Evans blue extravasation. Systemic VIP treatment did not prevent the albumin extravasation during the 30 min of the experiment, change of blue optical density was 61.2% and 57.1% in the vehicle- and VIP-treated animals, respectively. MO-evoked albumin-leakage was significantly decreased by maxadilan with 38.6% lower extravasation as compared to saline-treated animals.

These results were also confirmed by detecting the accumulation and fluorescence of ICG with *in vivo* imaging. Similarly to the Evans blue extravasation determined with intravital microscopy, neither maxadilan, nor VIP-treatment resulted in altered ICG-fluorescence in the PO-treated non-inflamed ears. In the MO-treated ears, maxadilan injection resulted in significantly reduced inflammatory hyperemia and vascular leakage with 34.3% lower ICG fluorescence as compared to the saline-treated mice. However, we could not detect any differences between the ICG-fluorescence of VIP- and saline-treated animals (Fig. 3).

3.3. Effect of VIP and maxadilan on 5% mustard oil-induced vasodilation in the mouse ear

Maxadilan induced a basal vasodilation in the mouse ear as indicated by the green color in the control images, while saline and VIP alone did not change the baseline perfusion (Fig. 4A, B, C). Neurogenic cutaneous vasodilation reached its maximum 6–10 min after the topical application of 5% mustard oil with a peak value of 87.4% in the vehicle-treated groups. Systemic administration of both the VPAC1/2 receptor agonist VIP and the PAC1 receptor agonist maxadilan significantly counteracted the MO-induced vasodilation with a maximum of 39.4% and 19.2% inhibition of the cutaneous blood flow, respectively. The inhibitory action of maxadilan was significantly greater compared to VIP. Interestingly, maxadilan diminished neurogenic vasodilation, although it acted as a potent vasodilator in the absence of MO-stimulation (Fig. 4).

3.4. Effect of VIP and maxadilan on the neutrophil recruitment

MPO activity, a quantitative indicator of neutrophil granulocyte function referring to the cellular components of the inflammation,

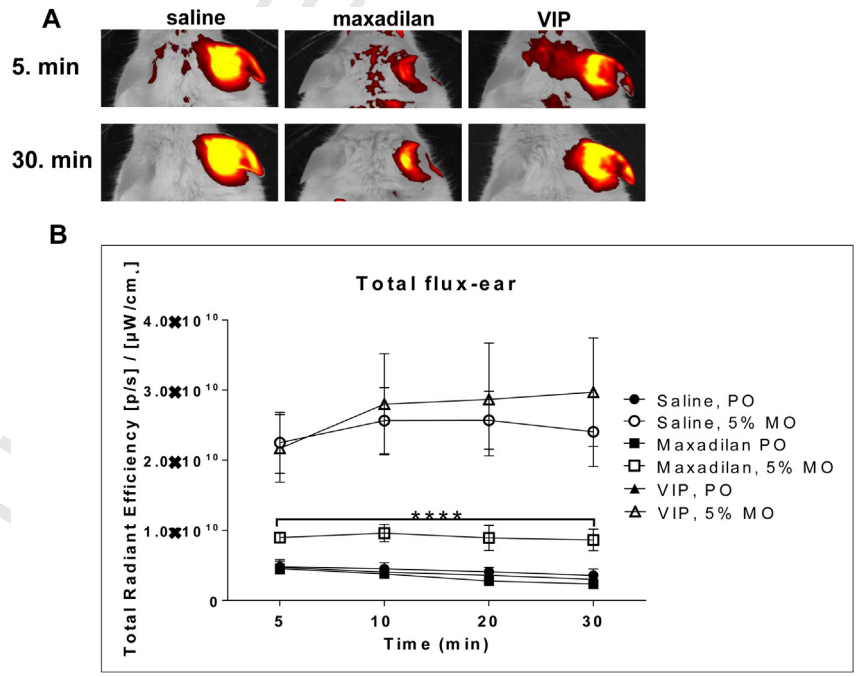

Fig. 3. Representative images of indocyanine green (ICG) fluorescence in the ear of saline-, maxadilan- and VIP-treated mice 5 and 30 min after mustard oil smearing and injection of ICG (0.5 mg·kg^{-1} i.v.) (A). Dark red color indicates areas with low blood flow, increasing perfusion is shown by light red and orange, while yellow color represent areas with the highest perfusion. Maxadilan-treated animals showed significantly lower ICG-fluorescence-increase in response to 5% MO, while VIP-treatment resulted in unaltered ICG-fluorescence as compared to vehicle-treated control animals. Self-controlled ICG-fluorescence intensity in the ears (B). Two-way ANOVA followed by Bonferroni's test; $n = 4–5$/group; ****$p < 0.0001$ vs. saline, 5% MO and VIP, 5% MO. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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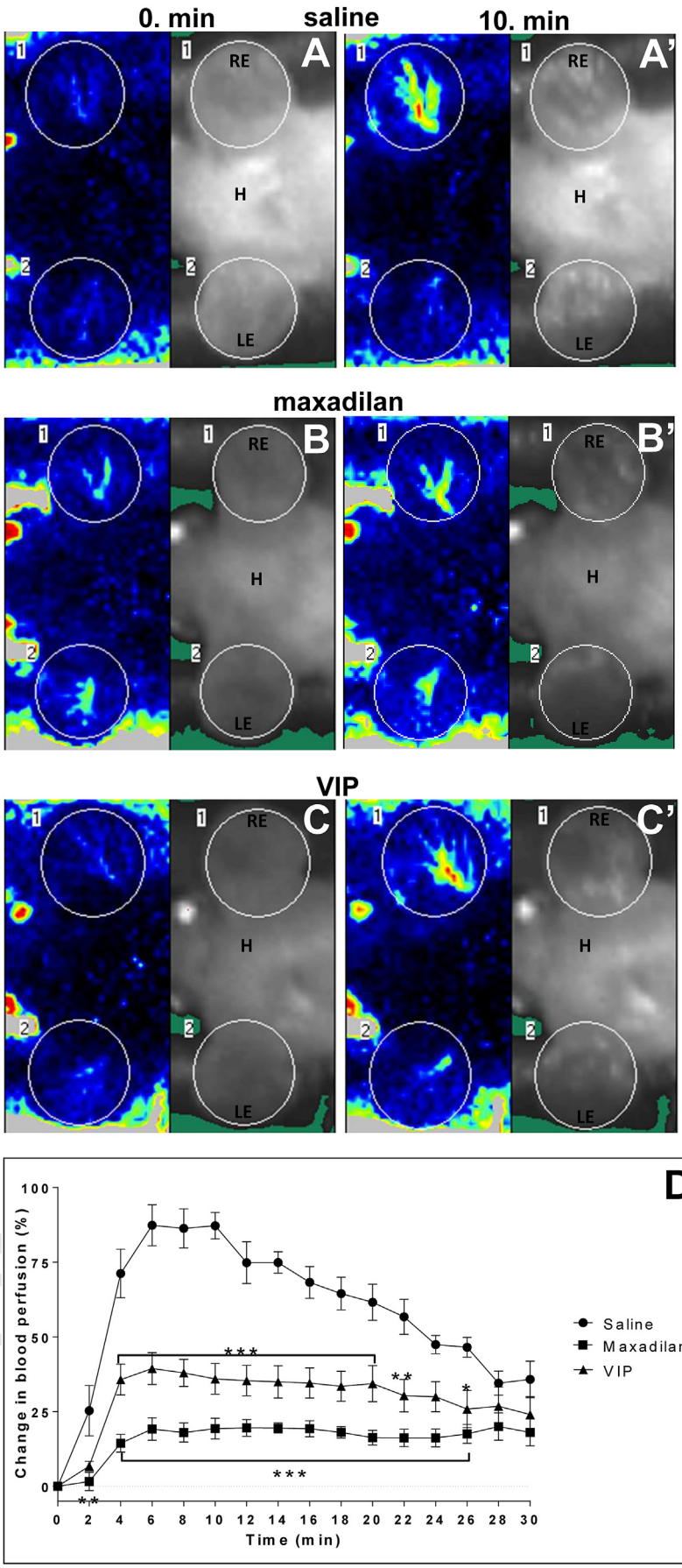

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was significantly increased in the mustard oil-treated ears 6 h after the MO-stimulation compared to the respective paraffin oil-treated controls. However, neither VIP, nor maxadilan exerted any effect on this parameter suggesting that activation of VPAC1/2 or PAC1 receptors does not influence the accumulation of granulocytes in either inflamed or non-inflamed tissues (Fig. 5).

3.5. Effect of maxadilan and VIP on the SP concentration of the mouse ear

Radioimmunoassay measurements revealed moderate, but significant increase in SP concentration of the mouse ear in response to 5% mustard oil smearing in the control, saline-treated animals. Maxadilan significantly inhibited the mustard oil-induced SP release from the sensory nerve terminals: no difference was observed between the mustard oil- and the paraffin oil-treated ears of the animals after i.p. maxadilan pretreatment. VIP did not influence the concentration of the peptide in the ears (Fig. 6).

4. Discussion and conclusions

In the present study, we provided the first evidence that activation of the PAC1 receptor by the selective agonist maxadilan inhibits acute neurogenic inflammation in the mouse. This effect is due to the inhibition of both arterial vasodilation and plasma protein leakage from the veins and capillaries as demonstrated by the significantly attenuated increase of tissue perfusion with laser Doppler imaging, as well as diminished plasma protein extravasation with micrometry, intravital microscopy and *in vivo* fluorescent imaging. Since maxadilan significantly decreased the MO-induced SP increase in the tissue, inhibition of sensory neuropeptide release from the activated peptidergic capsaicin-sensitive afferents is likely to explain these *in vivo* results. The edema-inhibiting action of the VPAC1/2 receptor agonist VIP was milder, reaching the level of statistical significance only 2 and 4 h after topical application of 5% MO, but not in case of swelling induced by 1% MO or 5% formalin. Edema formation is predominantly due to plasma protein extravasation, which was not influenced by VIP treatment. The later, basically non-neurogenic, cellular phase of the inflammation occurring 6 h after the topical application of MO in the mouse ear (Bánvölgyi et al., 2004) was not modified by either maxadilan or VIP as shown by similar MPO activities in the tissues. This finding is similar to that observed earlier for PACAP, which also decreased the neurogenic phase of the inflammation only, but had no effect on the infiltration of leukocytes (Németh et al., 2006).

Acute neurogenic inflammation is a key pathogenic mechanism of several diseases, including asthma, migraine and allergic contact dermatitis or rhinitis. Our results clearly show that PAC1 receptor agonists could be suitable candidates to inhibit the neurogenic components of these inflammatory diseases.

PACAP was shown to induce albumin extravasation in the rat skin and human nasal mucosa by stimulating histamin release, although it had no effect on ozone-induced albumin extravasation in the airways of guinea pigs (Aizawa et al., 1999; Cardell et al., 1997; Kinhult et al., 2003). Similarly to PACAP, maxadilan and VIP were also shown to trigger plasma leakage in intact tissues,

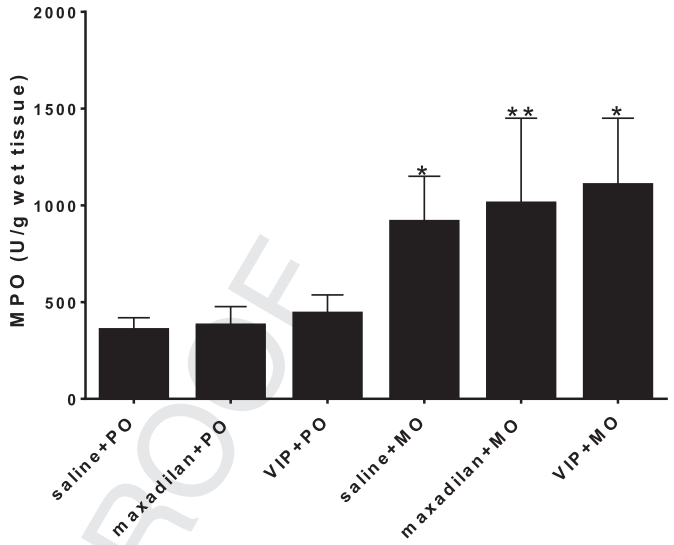

Fig. 5. Myeloperoxidase activity in the ear of i.p. saline-, maxadilan- and VIP-treated mice 6 h after topical application of paraffin or 5% mustard oil. No difference was found in the MPO activity between any of the paraffin- or mustard oil-treated groups. Columns represent means ± SEM; one-way ANOVA followed by Bonferroni's test; $n = 8–12$ per group; $^{*}p < 0.05$ saline + MO vs. saline + PO or maxadilan + MO vs. maxadilan + PO; $^{**}p < 0.001$ VIP + MO vs. VIP + PO.

implicating that in the absence of mustard oil stimulation, activation of both PAC1 and VPAC1/2 receptors is associated with increased vascular permeability (Inoue et al., 1993; Khalil et al., 1988; Svensjö et al., 2009, 2012). In an other study, Ro-24-99-81, a stable analogue of VIP, facilitated the vascular permeability induced by SP and capsaicin, while VIP had no effect (Gao et al., 1995). Stimulatory effect of VIP on neurogenic inflammation and mast cell degranulation is more pronounced than that of PACAP, suggesting the involvement of VPAC1/2 receptors (Schytz, 2010).

Data are also available on the opposite effect of PACAP, reporting that it reverses the increased permeability of postcapillary venules after electrical vagus stimulation or in case of SP-induced plasma leakage in the airways (Shigyo et al., 1998).

Németh et al. (2006) reported the concentration-dependent anti-inflammatory action of PACAP-38 on acute neurogenic inflammation, the neuropeptide was found to exert significant inhibitory action on MO-induced neurogenic edema and plasma albumin extravasation in a dose of 100 μg/kg. Moreover, PACAP exhibited a local immunomodulatory function by inhibiting capsaicin- and electrical field stimulation-induced release of CGRP, SP and somatostatin from the sensory nerve terminals of the trachea *in vitro*. Another study extended these findings, demonstrating that systemic stimulation of capsaicin-sensitive fibers by the TRPV1 receptor agonist resiniferatoxin leads to significant increase in the plasma concentration of PACAP in rats. Inhibitory action of PACAP against carrageenan-induced mixed-type inflammation was also demonstrated (Helyes et al., 2007). However, our present results are the first to reveal the receptorial mechanism responsible for the inhibitory effect of PACAP-38 in neurogenic inflammation.

Fig. 4. Representative laser Doppler images of both ears before (A, B, C) and 10 min after (D, E, F) the application of paraffin (left ear, marked by the lower circle in the picture) or 5% mustard oil (right ear, marked by the upper circle in the picture). Black and white images indicate the scanned area of the mouse head (marked by 'H' in the picture) and left and right ears (marked by 'LE' and 'RE' in the picture, respectively), while color photos show the blood perfusion. Control images of saline-treated (A), maxadilan-treated (B) and VIP-treated (C) animals. Images at the time of maximum vasodilation in the right ear from animals administered saline (A'), maxadilan (100 μg kg^{-1}) (B') and VIP (100 μg kg^{-1}) (C'). Black and blue color represents low perfusion areas, while green, yellow and red colors indicate areas with enhanced blood flow. Effect of VIP and maxadilan on 5% mustard oil-induced cutaneous vasodilation in the mouse ear (D). Both maxadilan and VIP treatment resulted in significant inhibition of the neurogenic inflammation caused vasodilation, however, maxadilan was found to be more effective. Two-way ANOVA followed by Bonferroni's test; $n = 7$ per group; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. saline. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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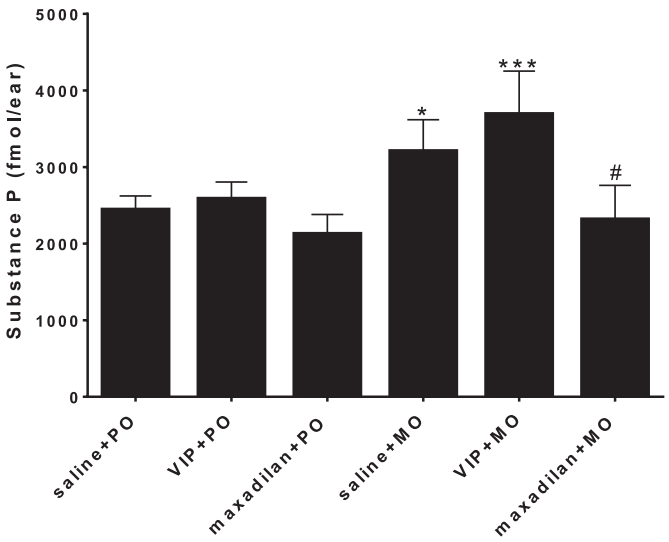

Fig. 6. Effect of maxadilan and VIP on the concentration of substance P (SP) in the mouse ear. I.p. administration of maxadilan significantly decreased the level of SP, while VIP treatment did not cause any changes in the SP concentration of the mustard oil-treated ears. Columns represent means ± SEM; one-way ANOVA followed by Bonferroni's test; $n = 5–11$ per group; $^{*}p < 0.05$ vs. saline + PO; $^{***}p < 0.001$ vs. VIP + PO; $^{\#}p < 0.05$ vs. saline + MO and VIP + MO.

Co-localization of PACAP with the structurally related VIP was reported in the parasympathetic and dorsal root ganglia, similarly to neurons innervating the lung, urogenital and gastrointestinal tracts (reviewed in Fahrenkrug and Hannibal, 2004). The presence of VIP-like immunoreactivity was also shown in the rat primary sensory neurons (Ju et al., 1987). Noguchi and coworkers found a significant loss in the number of VIP expressing neurons in the dorsal root ganglia of rats receiving capsaicin-treatment during the neonatal period (Noguchi et al., 1993). The anti-inflammatory actions of VIP are well-known, however, relatively little is reported regarding its involvement in neurogenic inflammation. Evidence has accumulated over the last decade that VPAC1 receptor is primarily responsible for the anti-inflammatory actions of VIP and PACAP in experimental arthritis and Crohn's disease, while PAC1R was found to mediate the protective effects against septic endotoxemia (Abad et al., 2003; Delgado et al., 2000, 2001; Martinez et al., 2006). Involvement of VPAC1/2 receptors was also reported in pressure-induced vasodilation, a process associated with the activation of capsaicin-sensitive nerve fibers and CGRP-release (Fizanne et al., 2004). Moreover, studies suggested that VIP is involved in several diseases, which are considered to develop as a result of neurogenic inflammation. Upregulation of VIP was detected in neurogenic inflammation in the rat retina (Bronzetti et al., 2007). Plasma VIP level of patients with acute asthmatic exacerbations was found to be significantly lower compared to the controls and showed correlation with the response to therapy, suggesting that bronchodilatory and vasodilatory actions of the peptide are essential in this regard (Cardell et al., 1994; Said, 1982). Even in a human study, VPAC2 receptor was shown to exert inhibitory action against bronchial asthma (Lindén et al., 2003). After conjunctival allergic challenge, VIP was found to be elevated in allergic patients similarly to the lesional skin of patients with atopic dermatitis, indicating an immunomodulatory action of the peptide (Giannetti et al., 1992; Sacchetti et al., 2011). Although, in atopic dermatitis, elevated VIP level is associated with more severe pruritus (Teresiak-Mikołajczak et al., 2013). Moreover, several studies proved its contribution to the non-adrenegric non-cholinergic relaxation (Lei et al., 1993; Van Geldre and Lefebvre, 2004). Similarly to other neuropeptides, VIP treatment was also shown to be effective against collagen-induced arthritis in mice (Niissalo et al., 2002). Based on our results, involvement of VPAC1 and 2 receptors in the inhibitory action of PACAP on neurogenic inflammation is limited only to arterial vasodilation.

A great amount of evidence proved the vasoregulatory effect and immunmodulatory properties of maxadilan in non-neurogenic inflammation, which were also shown to be essential for the transmission of Leishmania (Brodie et al., 2007). However, its involvement in neurogenic inflammation has not been investigated yet. Recently, Lauenstein et al. (2011) reported overexpression of PAC1 receptors in the lungs under inflammatory conditions, and its stimulation has been shown to result in markedly decreased number of eosinophils, implicating the crucial role of this receptor in the suppression of respiratory inflammation (Lauenstein et al., 2011). Our results demonstrate that despite the vasodilator effect of maxadilan – which was obvious in the control laser Doppler images of the paraffin-treated ears – under inflammatory conditions, PAC1 receptor activation diminishes sensory-nerve activation and neuropeptide-mediated increased blood flow. The presently known signaling mechanisms linked to PAC1 receptor stimulation all lead to cAMP and calcium increase, which cannot give an appropriate molecular explanation for the observed potent inhibitory actions on the sensory nerve endings. Since we have previously shown that PACAP6-38, which is widely used as a PAC1/VPAC2 antagonist in several systems, acts as a potent agonist on the sensory nerves (Reglodi et al., 2008), a not yet identified PAC1-related novel receptor or splice variant can be suggested on the capsaicin-sensitive afferents.

In summary, we found that maxadilan significantly attenuated neurogenic edema, reduced the increase of microcirculation and vascular permeability, as results of decreased SP release, while VIP treatment was only moderately effective in suppressing smaller neurogenic vasodilation. These data indicate that inhibitory effect of PACAP on the vascular changes of mustard oil-induced neurogenic inflammation is mediated via PAC1 receptors.

Acknowledgments

This study was supported by OTKA K104984, TAMOP 4.2.2.A-11/1/KONV-2012-0024, Arimura Foundation, PTE-MTA "Lendület" Program, Hungarian Brain Research Program – Grant No. KTIA_13_NAP-A-III/4 and KTIA_NAP-A-III/5. This research was realized in the frames of TAMOP 4.2.4. A/2-11-1-2012-0001, National Excellence Program – Elaborating and operating an inland student and researcher personal support system convergence program." The project was subsidized by the European Union and co-financed by the European Social Fund. Q4

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Please cite this article in press as: Banki, E., et al., The selective PAC1 receptor agonist maxadilan inhibits neurogenic vasodilation and edema formation in the mouse skin, Neuropharmacology (2014), http://dx.doi.org/10.1016/j.neuropharm.2014.06.019