Investigation of carbachol and PACAP38 in a human model of migraine

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THE FOUR PAPERS ARE:

- 1. The cholinomimetic agent carbachol induces headache in healthy subjects. Cephalalgia, 2009, vol. 29, p. 258-268.
- Carbachol induces headache, but not migraine-like attacks, in patients with migraine without aura. Cephalalgia, 2010, vol. 30, p. 337-45.
- 3. PACAP38 induces migraine-like attacks in patients with migraine without aura. Brain, 2009, vol. 132, p. 16-25.
- 4. Cutaneous nociception and neurogenic inflammation evoked by PACAP38 and VIP. J Headache Pain, 2010, vol. 11, p. 309-16

SPECIFIC AIMS OF THE THESIS

To explore the headache eliciting effect of the acetylcholine analogue carbachol in healthy subjects and patients with migraine without aura (MO).

To explore the headache eliciting effect of pituitary adenylate cyclase activating peptide-38 (PACAP38) in healthy subjects and patients with MO.

To study the nociceptive effects of VIP (vasoactive intestinal peptide) and PACAP38 in a cutaneous model of acute pain in healthy subjects.

INTRODUCTION

Migraine is one of the most prevalent neurological disorders with an estimated 41 million cases in Europe (1). The average 1-year prevalence of migraine in Europe is reported to be 17% in women and 8% in men and most prevalent from 20 to 50 years of age (2, 3). Migraine is on the WHO top 20 list of disabling disorders (4) and costs 27 billion euro per year in Europe (1).

Migraine is a complex neurological disorder whose mechanisms are being unravelled through various experimental approaches. Using animal models of migraine it is possible to explore vascular (5) and neuronal (6, 7) mechanisms and these models have contributed greatly to the understanding of migraine pathophysiology (8). However, these results need to be complimented by human experimental models as migraine by definition is a subjective human experience (9). In the last 20 years we have systematically investigated endogenous substances relevant to migraine in a human model of migraine (10). By this approach, signalling molecules capable of inducing migraine-like attacks have been identified (11-13) leading to development of new anti-migraine compounds (14, 15).

The overall aim of the present PhD thesis was to investigate acetylcholine, via the cholinomimetic analogue carbachol, and PACAP38 in a human model of migraine. In addition, we applied a cutaneous model of acute pain to explore if PACAP38 might induce pain, central sensitization, neurogenic inflammation and mast cell degranulation.

MIGRAINE PATHOPHYSIOLOGY

The key features of migraine attacks are an intense, pulsating, unilateral headache that is aggravated by movement of the head and accompanied by nausea, photo- and phonophobia (9). During migraine attacks 27 - 73% of patients also report autonomic symptoms, such as rhinorea, lacrimation and flushing (16, 17). The symptoms are believed to be driven by the trigeminoparasympathetic reflex via a four-neuron pathway: afferent trigeminal nociceptors \rightarrow trigeminal nucleus caudalis (TNC) neurons \rightarrow parasympathetic superior salivatory nucleus (SSN) neurons \rightarrow postganglionic sphenopalatine ganglion (SPG) neurons (18). Efferent fibres from postganglionic SPG neurons then innervate the lacrimal gland (19), nasal glands (20) and the anterior cerebral vasculature (21) (figure 1).



Figure 1 Illustration of the trigemino-parasympathetic reflex: afferent trigeminal nociceptors sending impulses to TNC neurons communicating with parasympathetic SSN neurons. Efferent fibres from postganglionic SPG neurons then innervate dural vessels and may release signalling molecules during migraine attacks. Modified from Goadsby et al (22), copyright © [2002] Massachusetts Medical Society. All rights reserved.

During migraine attacks perivascular nociceptors from the ophthalmic branch of the trigeminal nerve surrounding extracraniel, dural and pial arteries are likely to be activated (23). Activation of perivascular trigeminal nociceptors might initiate the trigeminoparasympathetic reflex and cause release of parasympathetic signalling molecules in the perivascular space. The SSN also receive input from various brain areas (18), such as the hypothalamus, and may be activated during onset of migraine (24). The precise role of parasympathetic signalling molecules in the perivascular space is unknown, but stimulation of trigeminal afferents increases intracranial blood flow via the SPG (25, 26). Furthermore, electrical stimulation of the SPG leads to ipsilateral plasma protein extravasation (PPE) in the rat dura mater (27). There is some human experimental evidence suggesting that parasympathetic activation might be pronociceptive, as migraine pain is reduced after anaesthetically blocking the SPG (28, 29), though others have failed to show an effect (30). The perivascular nerve fibres are found adjacent to mast cells, cephalic vessels and sensory neurons (31, 32) and could theoretically cause vasodilatation, PPE and mast cell degranunalation during migraine attacks. Thus, activation of the parasympathetic nervous system might not just be an epiphenomenon, but could serve as a feed-forward mechanism to trigger and amplify activation of perivascular nociceptors during migraine attacks (24).

ACETYLCHOLINE

ACETYLCHOLINE DISTRIBUTION

Acetylcholine exerts a multitude of actions in the brain. Acetylcholine is involved in vascular responses in cephalic vessels, direct neurotransmission in autonomic ganglions as well as cognitive processing, arousal and attention of the brain (33). This thesis discusses and investigates cholinergic mechanisms related to pain sensitive cephalic vessels.

The acetylcholine molecules in nerve fibres cannot be visualized by immunohistochemistry. However, staining of the enzyme acetylcholine-esterase (AChE), which converts acetylcholine into inactive metabolites, reveals dense plexuses of nerve fibres surrounding cerebral vessels in various animals (34). These cholinergic nerve fibres are believed to be of parasympathetic origin, as fibres are unchanged by prior cervical sympathectomy (34). AChE immunostained fibres have also been shown in human cerebral arteries (35), human middle meningeal arteries (MMA) (36, 37) and human superficial temporal arteries (STA) (38). A more selective marker of cholinergic fibres is the acetylcholine synthesizing enzyme choline acetyltransferase (ChAT) that stains nerve fibres surrounding human pial arteries and arterioles (39-41). Anatomical studies in the rat indicate that only one-third of the cholinergic innervation of the anterior cerebral vasculature originates in the SPG (21). Thus, cholinergic perivascular nerve fibres may also originate from the otic ganglion (OTG) and internal carotid ganglion (ICG) (figure 2) (42, 43).



Figure 2 Schematic representation of origins and pathways of cerebrovascular ChAT immunoreactive nerves in the rat. ACA: anterior cerebral artery; MCA: middle cerebral artery; ICA: internal carotid artery; PCA: posterior cerebral artery; BA: basilar artery. Reprinted from Suzuki et al (43), with permission from Nature Publishing Group.

ChAT has also immunocytochemically been localized in cells of small vessels (44), capillaries (45, 46) and arterioles (45) of the rat brain and in porcine microvessels (47). However, less than 10 % of endothelial cells have ChAT immunoreactivity in rat cortical capillaries and small blood vessels (44). In rabbit pial vessels, denudation of endothelium does not affect ChAT activity implying a neuronal origin of the activity (48). ChAT activity in cephalic vessels has not been well investigated in man. Endothelial cells from human fetal brain capillaries do not have ChAT activity (49), whereas retinal vessels show ChAT activity (50). Thus, whether acetylcholine synthesized in non-neuronal endothelial cells has any physiological role is yet to be determined.

ACETYLCHOLINE RECEPTORS AND FUNCTION

The acetylcholine receptors consist of muscarinic and nicotinic receptors. Five muscarinic receptors have been identified (M1-M5), which are G protein-coupled (51, 52). Odd numbered muscarinic receptors (M1, M3 and M5) activate phospholipase C, while even numbered muscarinic receptors (M2 and M4) inhibit adenylat cyclase (53, 54).

Nicotinic receptors are members of the ligand-gated ion channel superfamily and are pentamers consisting of five subunits. The neuronal nicotinic receptors exist in various combinations of seven α -like subunits and three β -like subunits (55). Binding of acetylcholine and other agonists cause opening of the central aqueous pore through which Na+, Ca2+ or K+ permeate to increase neuronal excitability or modulate neurotransmitter release (56, 57).

Acetylcholine dilates human cephalic arteries through muscarinic receptors located on the endothelium (35, 36, 38). Binding to these receptors activates endothelial nitric oxide (NO) synthase (eNOS) (58) and stimulates production of NO, a well known trigger of headache (11). M1 and M3 receptors are located on human pial vessels (59). However, under the exact same experimental conditions the M2 receptor cannot be identified (59), even though it is present on cat pial vessels (59). It is well documented that acetylcholine induces dilatation of human cephalic arteries in a wide range of concentrations (10-11 - 10-4 M) (36, 38, 60, 61). In cat cerebral vessels acetylcholine induces dilatation at low concentration (< 10-5 M) via endothelial M3 receptors, but constriction at high concentration (≥ 10-5 M) via vascular smooth muscle cell (VSMC) M1 receptors (62-64). Thus, important species differences in muscarinic receptors exist. This should lead to caution when interpreting study results.

Other muscarinic receptor subtypes might be involved in modulation of cephalic vessels as well. In monkey and porcine cerebral artery the actions of NO containing nerve fibres are inhibited by acetylcholine acting via prejunctional M2 receptors, suggesting an inhibitory regulatory mechanism (65, 66). Nicotinic receptors might be indirectly involved in dilatation of cerebral vessels. Thus, activation of α 7-nicotinic receptors on sympathetic nerve terminals cause release of norepinephrine resulting in production of NO and vasodilatation in porcine BA via presynaptic b2adrenoceptors located on adjacent nerve terminals (67, 68). In summary, acetylcholine released from parasympathetic nerve endings can change cephalic vessel tone via several mechanisms.

Other experimental data relevant to the present thesis have shown that acetylcholine can activate nociceptive C-fibres in animals (69, 70) and humans (71). The cholinomimetic agent carbachol is also an effective inducer of mast cell degranulation (72-74) including dural mast cells (31).

In study I & II these possible effects will be investigated using carbachol, since acetylcholine is quickly degraded by cholinesterases and therefore practically impossible to administer in a

human migraine model. Carbachol is not metabolized by cholinesterases which makes it suitable for exogenous administration. Carbachol does not pass the blood-brain-barrier (BBB) (75) and therefore the present studies reflect cholinergic effects on brain blood vessels from the luminal side, whereas the effects on dural and extracranial vessels are from both the luminal and abluminal side since they are devoid of BBB.

ACETYLCHOLINE IN RELATION TO MIGRAINE

Inhalation of the cholinergic agonist methacholine has previously been reported to cause headache in one subject (76) and methacholine induced increases in plasma cyclic guanosine monophosphate (cGMP) was much larger in patients with migraine than in healthy subjects (77). However, cholinergically induced headache and its effect on cephalic hemodynamics in humans in vivo have never previously been systematically studied in man.

PACAP38

PACAP DISTRIBUTION

PACAP is found both as a 38 amino-acid peptide (PACAP38) and as a truncated 27 amino-acid peptide (PACAP27). PACAP belongs to the same glucagon/secretin superfamily as the 28 amino-acid peptide VIP, where the N-terminal 28 amino-acids of PACAP38 share 68% homology with VIP (78). PACAP38 is the most predominant as PACAP27 represents less than 10% of the total PACAP content in most tissues, including the CNS (79-82). PACAP38 is distributed throughout the body and has a wide range of biological functions (83). This thesis will focus on the involvement of PACAP38 in cephalic vasodilatation and pain.

PACAP immunoreactive nerve fibres innervate cerebral vessels in mammals (84-86). PACAP has been identified in human sensory (87) and parasympathetic (88) ganglia as well as in the TNC (89). VIP and PACAP are co-localised in rat parasympathetic ganglia (86, 90) and cat cerebral vessels (84). VIP and PACAP38 immunoreactive nerve fibres are also present in human skin close to mast cells, sweat glands and hair follicles (91-95).

PACAP RECEPTORS AND FUNCTION

The action of VIP and PACAP is mediated through three G proteincoupled receptors: VPAC1 and VPAC2 receptors that have an equally high affinity for VIP and PACAP, and the PAC1 receptor which has a 1,000 fold higher affinity for PACAP than for VIP (96). Activation of all receptors increases cyclic adenosine monophosphate (cAMP), but also phospholipase C (PLC) and intracellular calcium have been reported as effector pathways (figure 3) (97, 98).



Figure 3 Highlight of the principal intracellular signalling pathways activated by VPAC1, VPAC2 and PAC1 receptors. Upon activation, all three receptors are capable of causing downstream production of cAMP. In addition, the three receptors can also activate PLC leading to an increase in Ca2+ (97). Reprinted from Dickinson et al (98), with permission from Elsevier.

The mRNA for all VIP/PACAP receptors are found in human cerebral arteries (99), sensory, parasympathetic and in sympathetic ganglia with perivascular nerve fibre projections (99). PACAP38 elicits vasodilatation in animal (84, 100, 101) and human (102) cerebral arteries. In human skin PACAP38 degranulates mast cells and causes histamine release in vitro (95). PACAP38 might also have a role in central pain transmission (103).

PACAP38 IN RELATION TO MIGRAINE

It has previously been shown that VIP induces a marked dilatation of cephalic arteries, but only mild headache and no migraine-like attacks (104, 105). Infusion of PACAP38 induces vasodilatation in healthy subjects of a similar magnitude but is longer lasting than VIP (102). However, the headache characteristics after PACAP38 have not been previously described in healthy subjects or migraine patients.

METHODS

VOLUNTEERS

Healthy subjects between 18 - 45 years of age were recruited for study I, III and IV. Exclusion criteria were: a history of migraine or any other type of headache (except episodic tension- type headache < 5 days/month); any daily medication apart from oral contraceptives; serious somatic or psychiatric disease. Migraine patients in study II and III were 18 - 47 years old volunteers diagnosed with MO according to the International Headache Society (IHS) (9). Exclusion criteria were as in study I with the exception of the MO diagnosis. All subjects gave informed consent to participate in the studies that were undertaken in accordance with the Helsinki Declaration of 1964, as revised in Edinburgh in 2000.

EXPERIMENTAL DESIGN STUDY I-III

All three studies were conducted using a balanced randomised double-blind, placebo controlled crossover design. In study I-II volunteers were randomly allocated to receive 3 µg/kg carbachol or placebo over 25 min on two days, separated by least 7 days. In study III all volunteers were randomly allocated to receive 10 pmol/kg/min PACAP38 or placebo over 20 min on two days, separated by at least 7 days. All subjects reported to the laboratory at 0830 h headache free. After baseline measurements the infusion started using a time and volume controlled infusion pump. Headache intensity, accompanying symptoms, velocity of the middle cerebral artery (VMCA), diameter of the STA, diameter of the radial artery (RA) (study I), end-tidal partial pressure CO2 (PetCO2), adverse events and vital signs were recorded at baseline and then every 10 min until 90 min from the beginning of infusion (figure 4, p. 24).

The subjects were carefully instructed to complete a headache diary with accompanying symptoms according to the IHS (106)

and state use of rescue medication every hour until 12 h after discharge from the hospital. Subjects were allowed to take rescue medication of their own choice at any time.

Headache

Headache intensity was recorded repeatedly on a verbal rating scale (VRS) from 0 to 10 [0, no headache; 1, a very mild headache (including a feeling of pressing or throbbing – pre-pain); 10, worst imaginable headache] (107). Headache characteristics and associated symptoms were also recorded to determine the quality and type of the headache.

Experimental headache induced by infusion of a neurotransmitter cannot fulfil strict IHS criteria for MO (9). First, the migraine-like attacks reported are induced by pharmacological substances and can therefore not be spontaneous, though they phenotypically mimic spontaneous migraine attacks in the majority of patients (11, 13). Secondly, most spontaneous migraine attacks develop in a matter of hours and often go through a phase where they phenomenologically fulfil the criteria for tension-type headache before the headache gets worse, becomes unilateral and has the associated symptoms required for migraine. For this reason attacks aborted by migraine specific treatment before fulfilling all criteria for migraine were accepted in the new criteria for chronic migraine (108). Patients in experimental provocation studies cannot be denied treatment of the induced attacks and often treat before all migraine criteria are fulfilled.

Based on these considerations the following two criteria for a migraine-like attack induced 0-12 h after infusion of an experimental drug were used:

Migraine-like attacks fulfilling either 1 or 2:

1. Headache fulfilling criteria C* and D for MO (9).

2. Headache described as mimicking usual migraine attack and treated with a triptan.

*Moderate to severe pain intensity is considered \geq 4 on the VRS.

Cerebral hemodynamics

Changes in large cerebral artery diameter are not easily investigated in humans in vivo. Velocity is dependent on the absolute flow and the cross-sectional area of the artery. Thus, changes in velocity indicate changes in the diameter only when the absolute flow is unchanged (109, 110). Due to technical reasons it was only possible to do single photon emission computer tomography (SPECT) in half of the subjects in study I and in the healthy subjects in study III, which has been published elsewhere (102). Changes in MCA diameter in study I were calculated according to the following equation (110):

 $\Delta d = (\sqrt{(rCBFMCA(b) / VMCA(b)) \times (VMCA(a) / rCBFMCA(a)))-1} \times 100$

In study II & III relative changes in MCA diameter, since rCBF was assumed to remain unchanged, were calculated as: $\Delta d = (\sqrt{VMCA(a) / VMCA(b)} - 1) \times 100$

rCBF: regional cerebral blood flow in the MCA area.

Transcranial Doppler and PetCO2

Blood flow velocity in the MCA was selected because recordings of VMCA are the most reproducible with the present methodology, with a day-to-day coefficient variation of 16% and a 5 min coefficient variation of 7 %. VMCA was recorded bilaterally by transcranial Doppler ultrasonography (TCD) as previously described (104, 111). The average of four measurements, comprising approximately four cardiac cycles, each over a time interval of 16 s was used. A fixed point for measurements of VMCA was chosen along the MCA as closely possible to the bifurcation between the MCA and ACA. For each individual this fixed point was used on both study days, and every measurement was done after carefully optimizing the signal from this point. Henrik Winther Schytz (HWS) performed all the Doppler recordings. PetCO2 was recorded simultaneously to the TCD measurement using an open mask, not causing respiratory resistance. Hypocapnia decreases VMCA due to changes in arteriolar tone. VMCA values was therefore post-processed and corrected for significant changes in PetCO2 by e0.034 for each mmHg decrease in PetCO2 (112).

Cerebral blood flow and PetCO2

Cerebral blood flow (CBF) and rCBFMCA measurements were done with 133Xe inhalation SPECT, which is a suitable and reproducible method for assessing absolute changes (113). SPECT has a day-to-day coefficient of variation of 8% (114) which makes it slightly better than magnetic resonance imaging and approximately equal to positron emission tomography (115). The examination was performed with the patient in the supine position with eyes closed. Four markers were drawn on the skin to ensure accurate positioning in each acquisition. PetCO2 was measured during each examination. CBF was measured with 133Xe inhalation using a brain-dedicated SPECT gamma camera. The system uses a stationary annular NaI crystal and a fast rotating collimator. Flow was calculated in each pixel based on the clearance curve, output was the ki value (116). To obtain CBF values, a partition coefficient (λ) of 0.85 was used. Calculation of flow in the perfusion territories of the major cerebral arteries was performed by fitting standard vascular region of interest on 5 axial slices of the brain positioned 3.6, 5.0, 6.3, 7.6, and 9 cm above the orbito-meatal (OM) plane (117). During CBF measurements PetCO2 was recorded by means of a capnograf. Hypocapnia decreases CBF (118). CBF values were therefore corrected for significant changes in PetCO2 by 2% for each mmHg change in PetCO2 (118).

Dermascan C

The diameter of the frontal branch of the left STA was measured in all studies. The diameter of left RA was, due to practical reasons, measured only in study I. Measurements were performed by a high-resolution ultrasonography unit. Diameter recordings were based on the mean of four measurements within the shortest possible time interval (less than 1 min) at each time-point (119) and taken at arbitrary time points in the cardiac cycle. Marks were drawn on the skin to ensure that repeated measurements were performed in the same place. The positions of measurements in relation to the angle and distance relative to the OM plane for the STA and the distal volar crest of the wrist for the RA were recorded to ensure reproducibility in measurements from day to day. The same skilled and trained lab technician performed the measurement for each subject. With the used technique and design the measurements have a day-to-day coefficient of variation of 12% (119, 120).

Vital signs and adverse events

Heart rate (HR) and blood pressure were measured with an autoinflatable cuff. Electrocardiogram was monitored on a screen and recorded on paper.

Blood pressure was measured on the left arm before scanning the RA. During the in-hospital phase the subjects were questioned for the presence of any adverse events by the investigator every 10 min.



Figure 4 Illustration of the human migraine model. Experimental drugs were infused into the antecubital vein. Headache intensity according to the VRS, accompanying symptoms and hemodynamic variables were recorded at 10 min intervals for 90 min. Ultrasound devices measure RA diameter, VMCA and STA diameter as shown from left to right. Reprinted from Olesen et al (121), with permission from Wiley-Blackwell.

Statistical analysis

All values were presented as mean values \pm SD, except headache scores, which were presented as median values. Peak mean vascular variables were presented with 95% confidence intervals. Baseline was defined as TO before the start of infusion of each dose.

The plasma half-life of carbachol is not known, whereas the plasma half life of PACAP38 has been shown to be 3.5 min in humans (102). We defined an infusion phase as 0 to 30 min, a post-infusion phase from 30 to 90 min and a post-hospital phase 2-12 h after start of infusion in study I-III.

Incidence of headache, migraine-like attacks and adverse events were analyzed as binary categorical data with McNemar test. Area under the curve (AUC) was calculated according to the trapezium rule (122) to obtain a summary measurement and to analyze the differences in response between active drugs and placebo. Baseline was subtracted before calculating AUC to reduce variation between sessions within subject. Analysis of AUC values were performed with a paired two-way t-test, except headache scores where data were tested with Wilcoxon signed rank test. We tested for period and carry-over effects for all baseline variables with Mann-Whitney test and independent t-test.

For CBF, PetCO2 and VMCA values (study I) as well as systolic blood pressure, diastolic blood pressure and mean arterial blood pressure (MAP) values (study I & III) continuous variables were analyzed for changes over time for each dose separately with univariate analysis of variance with the fixed factors volunteer and time. If overall differences were found, Dunnett's test was applied to characterize which time points were different from baseline.

All analysis was performed with SPSS. Five percent (P < 0.05) was accepted as the level of significance.

EXPERIMENTAL DESIGN STUDY IV

In a double-blind placebo-controlled crossover design subjects were, in a balanced order, randomly allocated to receive injections of PACAP38 and placebo or VIP and placebo on two study days separated by at least two days. Thus, each subject received two injections each day, active in one and placebo in the other arm. HWS performed all the experimental procedures, while the subjects rested in a supine position on a comfortable bed in a quiet room (temperature of 24–25 °C). Prior to provocation, participants rested in the supine position for at least 30 min to reduce influences of experimental conditions on cutaneous blood flow (123). Likewise, intake of coffee, tea, cocoa, tobacco, methylxanthine-containing foods or beverages was not allowed for the last 4 h before the study (123).

Baseline assessments for sensitivity to light brush (alloknesis and allodynia) and punctuate pain threshold with von Frey hair (VFH) were performed before the experimental drug injection. Spontaneous pain intensity was recorded after the experimental drug injection. Assessment of flare, skin blood flow and wheal were recorded 5, 15 and 20 min after injection of the experimental drug. Thereafter, assessment of brush-evoked allodynia and alloknesis, punctuate hyperalgesia and pain threshold were performed in the named order 5 and 20 min after the injection. The validated Danish version of the McGill pain questionaire (MPQ) (124) was then used to assess the subjective pain quality and pain distribution.

Intradermal injections

The test substrates were $18.1 \,\mu$ g/ml PACAP38 and $13.3 \,\mu$ g/ml VIP to produce 200 pmol PACAP38 and 200 pmol VIP. Placebo was isotonic saline. All experimental drugs were prepared and randomized by the Central Pharmacy, Herlev Hospital, Denmark. The subjects arm was placed in a comfortable position and 0.05 ml was injected intradermally at an angel of approximately 15 degree using a 29-gauge needle.

The injection site was the flexor side of the forearm 1/3 distal to the cubital fossa, no more than 1 cm off midline avoiding visible veins. This injection point was chosen since proximal sites of the human forearm show the most reproducible and robust responses to pain activation (125). In the opposite arm, the other experimental drug was injected similarly.

Assessment of pain intensity, distribution and quality

Experimental drug-induced pain intensity was recorded continuously every 2 s on a 10 cm electronic visual analogue scale (VAS), with anchor points 0 = no pain and 10 = the worst imaginable pain. Data were collected in a computer for later analysis. Subjects did not see the numeric correlates to their pain perceptions at any point during the study. Experimental drug induced pain was determined and expressed as area under the VAS-time curve (VASAUC) in cm x min, peak pain intensity and pain time. When testing and recording of the investigated arm were completed, the subject chose the appropriate descriptor words of the MPQ and was asked to draw the perceived pain distribution on an armchart. The pain distribution area was quantified later by cutting and weighing the pain drawing using an analysis weight (readability 0.1 mg).

Measurement of brush-evoked allodynia and alloknesis

The area of allodynia and alloknesis, using a soft brush, was assessed 5 and 20 min after the injection. The test was performed while the subjects kept their eyes closed. The borders of allodynia and alloknesia were determined by brushing along eight radiating lines at 45 angles of 6 cm length originating from the injection site.

Brushing was started from the periphery well outside the area and gradually moved towards the injection site in steps of 1 cm at intervals of 2 s. The borders were identified when the subjects reported the point, at which there was a clear transition from non-painful normal sensation to pain (burning, tender or unpleasant sense) (allodynia) or a distinct feeling of itch (alloknesis). On the basis of the marked points traced on an acetate sheet, polygons were drawn and the areas were calculated, thus polygons for the allodynic and alloknesic area were assessed (126).

Assessment of secondary hyperalgesia

The area of punctuate hyperalgesia was assessed 5 and 20 min after injection. The test was performed while the subjects kept their eyes closed. A handheld VFH nylon monofilament (No. 17, bending force 60.0 g) was used (127). The borders of the area were determined at a point along 8 spokes radiating at 45 degree angles of 6 cm length originating from the injection site. Stimulation was started from the most outside points towards the injection site in steps of one cm with an interval of 2 s. The border of the secondary hyperalgesic zone was identified when the subjects reported that the VFH stimulation changed to a greater pain sensation. Based on the marked sites traced on an acetate sheet, a polygon was drawn and the area was calculated (128).

Assessment of skin blood flow

Forearm skin blood flow was measured by laser Doppler flowmetry, which is a standard real-time method measuring blood flow in the blood vessels of the microvasculature (129). Relative blood flow changes (expressed in %) were calculated by subtracting the baseline blood flow from blood flow at 5, 15 and 20 min after injection of the experimental drug. An area of 4 x 4 cm2 was scanned with the injection in the center of the area at a distance of 30 cm from the skin. The image resolution was obtained at 114 x 114 pixels with a scan speed of 4 ms/pixel. Each single scan lasted 69 s. The use of laser Doppler flowmetry has contributed to quantitative evaluation of neurogenic inflammation in terms of blood flow (130-132) and assessment of superficial blood flow (133).

RESULTS

Assessment of flare

Flare (erythema around the injection site) was identified visually by HWS and mapped 5, 15 and 20 min after the injection on an acetate sheet. The flare areas were subsequently quantified by cutting and weighing the maps on a laboratory weight (readability 0.1 mg). The maximal diameter (in cm) was assessed 2, 4 and 6 h after each injection by each subject.

Assessment of wheal

The wheal (the area of oedema caused by plasma extravasation) was inspected visually and by light palpation by HWS 5, 15 and 20 min after the injection. Afterwards the wheal was outlined with a marker pen on transparent removable tape above the wheal and the tape was pasted on millimetre paper and later calculated to total wheal area (in mm2).

Von Frey hair stimulation

The pain threshold for tactile stimulation was determined using a set of calibrated VFH. Each VFH was assigned a number representing a logarithmic increase in pressure. The stimulation was applied within 0.5 cm from the injection point. The participants were blinded for the VFH number and were stimulated three times with a frequency of 0.5 Hz (2 s), with each VFH applied in random order.

The subjects were instructed to report how many of the stimulations felt painful like a pinprick (0-3). The smallest VFH number capable of inducing pain in two of three trials was considered threshold (134).

Statistical analysis

The pain score results are expressed as median values with quartiles. Other variables are expressed mean values \pm SD. Given that the data were not normally distributed a Friedman's two-way analysis of variance were performed for the four groups: PACAP38, VIP, placebo on PACAP38 day and placebo on VIP day. If the overall P value was less than 0.05, we applied a post hoc Wilcoxon signed rank test to test the differences between relevant groups. PACAP38 was compared to placebo on the opposite arm on the injection day and the same was done for VIP, since it has been shown that arm-to-arm comparison is more reproducible than period-to-period comparison (125). Five percent (P < 0.05) was accepted as the level of significance.

STUDY I: CARBACHOL IN HEALTHY SUBJECTS

Infusion of carbachol induced headache in 9 out of 12 healthy subjects compared to 3 out of 11 after placebo (P = 0.063) (figure 5) (135). The AUC for headache was increased after carbachol compared to placebo, both during infusion (AUC0-30 min, P = 0.042) and in the post-infusion phases (AUC30-90 min, P = 0.027). Median headache intensity (1.0) peaked at 40-50 min after start of carbachol infusion. No difference was found between carbachol and placebo in the AUC in the post-hospital phase (AUC2-12 h, P = 0.225).



Figure 5 Individual (black lines) and median (thick red lines) headache scores obtained on a VRS from time 0-90 min and 2-12 hours in 12 healthy subjects. Black arrows show infusion.

Peak vascular responses after carbachol are seen in table 1. Analysis of variance showed no changes in global CBF (carbachol, P =

0.145, placebo P = 0.111) or rCBFMCA (carbachol, P = 0.162, placebo P = 0.180) over time. VMCA decreased on carbachol compared to placebo during the infusion (P = 0.003) and the post-infusion (P = 0.029) phases. There was an increase in STA diameter on carbachol compared to placebo during the infusion (P = 0.006) and post-infusion phases (P = 0.020). There was no difference in RA diameter on carbachol compared to placebo during any phases in the hospital period (P > 0.05). Adverse events included increased saliva, lacrimation, heat sensation and sweating and were more often reported on carbachol than on placebo.

	Peak time (min)		Mean change from baseline (95% confidence interval)	
Variable	Healthy	мо	Healthy	МО
MCA diameter	20	20	5.2 % (2.3 – 8.1)	4.5 % (2.4 – 6.7)
VMCA	20	20	-8.8 % (-13.4 – -4.2)	-8.0 % (-11.8 – -4.2)
STA diameter	10	30	18.8 % (8.9 – 28.7)	7.2 % (2.9 – 11.6)
Heart rate	20	20	24.4 % (18.5 – 30.3)	14.7 % (8.9 – 20.4)
MAP	10	10	-5.5 % (-8.6 – -2.4)	-3.4 % (-5.1 – -1.7)

Table 1 Mean peak hemodynamic variables 0-90 min after carbachol infusion in 12 healthy subjects and 18 migraine patients. Healthy: healthy subjects. MO: migraine without aura patients. MCA: middle cerebral artery. VMCA: velocity MCA. MAP: mean arterial blood pressure.

STUDY II: CARBACHOL IN MIGRAINE WITHOUT AURA PATIENTS In total 15 out of 18 MO patients experienced headache (0-12 h) after carbachol infusion compared to 8 out of 18 after placebo (P = 0.039) (figure 6) (136). Thirteen subjects experienced headache during the hospital phase on carbachol day compared to 3 on placebo day (P = 0.002). During the post-hospital phase 11 subjects experienced headache compared to 7 on placebo day (P = 0.344).



Figure 6 Individual (black lines) and median (thick red lines) headache scores obtained on a VRS from time 0-90 min and 2-12 hours in 18 migraine patients. Black arrows show infusion. There was no difference in incidence of migraine-like attacks after carbachol (n = 8) compared to placebo (n = 6) (P = 0.687). The median peak headache score was 2 (range 0 - 8) after carbachol. The median time to peak headache after carbachol was 1.0 h (range 0-12).

During the hospital phase AUC for headache was larger after carbachol compared to placebo during the infusion (AUC0-30 min, P = 0.012) and the post-infusion phases (AUC30-90 min, P =0.028). There was no difference in AUC between carbachol compared to placebo (AUC1.5-12 h, P = 0.972) in the post-hospital phase.

The peak responses of vascular variables after carbachol are shown in table 1 and figure 7. VMCA decreased after carbachol compared to placebo during the infusion (P = 0.044), but not during the post-infusion phase (P = 0.533). There was no difference in STA diameter on carbachol compared to placebo during infusion (P = 0.089) or post-infusion phases (P = 0.779). Adverse events included increased saliva, sweating, urge to void, lacrimation and heat sensation were more often reported on carbachol than on placebo.



Figure 7 Hemodynamic changes from baseline and median headache score obtained by a VRS in migraine patients on carbachol day.

STUDY III: PACAP38 IN HEALTHY SUBJECTS AND MIGRAINE PA-TIENTS WITHOUT AURA

PACAP38 infusion caused headache in all healthy subjects (P = 0.002) and 11 out of 12 migraine patients (P = 0.021) (figure 8) (137). Seven migraine patients experienced migraine-like attacks after PACAP38 and none after placebo (P = 0.016). Most of attacks (6 out of 7) occurred during the post-hospital phase (mean time 6 h [range 2-11]). Two healthy subjects reported migraine-like attacks after PACAP38 during the hospital phase and none during the post-hospital phase.



Figure 8 Individual (black lines) and median (thick red lines) headache scores obtained on VRS from time 0-90 min and 2-12 hours in 12 healthy subjects and 12 migraine patients. Black arrows show infusion.

For the healthy subjects the median peak headache score was 3.5 (range 2-4) and the median time to peak headache was 5.5 h after infusion start of PACAP38.

For MO patients the median peak headache score was 2.5 (range 0 - 10) and the median time to peak headache occurred at a median of 4.0 h after infusion start of PACAP38. In the hospital phase, the AUC for headache score was larger during PACAP38 infusion compared to placebo in healthy subjects (P = 0.005) and tended to be larger in migraine patients (P = 0.066). In the posthospital phase, the AUC for headache was larger after PACAP38 infusion compared to placebo in both healthy subjects (P = 0.005) and migraine patients (P = 0.013).

In migraine patients, PACAP38 caused a peak decrease of 16.1% in VMCA and a 37.5% increase in STA diameter at 20 min after start of infusion (figure 9). Adverse events included heat sensation, palpitations and flushing and were more often reported on PACAP38 than on placebo in both healthy subjects and migraine patients.



Figure 9 Hemodynamic changes from baseline and median headache score obtained by a VRS in migraine patients on PACAP38 day.

STUDY IV: PACAP38 AND VIP IN A CUTANEOUS PAIN MODEL Pain after PACAP38 and VIP was mild and limited to a short time of about 100 s after the injection (figure 10) (138).



Figure 10 Median VAS pain scores following injection of 200 pmol VIP and 200 pmol

PACAP38. Median pain score did not exceed 0 on any placebo day.

The VASAUC was larger following VIP (P = 0.01) and PACAP38 (P = 0.004) than after placebo (table 2). The pain distribution area was larger after VIP (P = 0.023) and PACAP38 (P = 0.001) than after placebo (table 2). No statistical difference was found between VIP and PACAP38.

	VIP	PACAP38	Placebo
VASAUC (cm x s)	1.2	1.0	0.2
	(0.6-1.7)*	(0.4-1.6)*	(0-0.4)
Pain distribution	5.3	2.9	1.6
area (cm2)	±8.0*	±5.3*	±3.5
Peak pain (VAS)	1.3	1.7	0.5
	(0.9-2.8)	(1.0-2.2)	(0-1.2)
Pain time (s)	96	63	26
	(83-136)	(49-120)	(2-59)

Table 2 Median values with quartiles, except pain distribution asmean (\pm SD). *P < 0.05 for active drug vs. placebo. Placebo values</td>are a mean of placebo on VIP and PACAP38 day. Peak pain andpain time were not analyzed statistically.

The area of alloknesia and allodynia were not dependent on any of the injected substances (P > 0.05). The area of punctuate hyperalgesia was larger after VIP injection than after placebo (P = 0.006), and after PACAP38 compared to placebo (P = 0.011). No statistical difference was found between VIP and PACAP38 at any time points.

The maximal increase in skin blood flow was larger after VIP (396 \pm 178%) and PACAP38 (245 \pm 144%) compared to placebo (50 \pm 48%) 5 min after injection (P < 0.001) (figure 11). VIP induced maximal increase in skin blood flow was larger than PACAP38 (P = 0.002).

The maximal flare area was larger after VIP (7.4 \pm 3.8 cm2) compared to placebo (0.4 \pm 0.3 cm2) 5 min after injection (P < 0.001). The flare area was also larger after PACAP38 (3.4 \pm 2.2 cm2) compared to placebo (0.4 \pm 0.3 cm2) 5 min after injection (P < 0.001). VIP increased skin blood flow more than PACAP38 (P = 0.001) at 5 min after injection.

The maximal wheal area was larger after VIP (95 \pm 93 mm2) compared to placebo (2 \pm 4 mm2) 20 min after injection (P = 0.001). The maximal wheal area was also significantly larger after PACAP38 (24 \pm 37 mm2) compared to placebo (4 \pm 11 mm2) 20 min after injection (P = 0.011). The VIP induced maximal wheal area was larger than PACAP38 (P = 0.001).



Figure 11 Example of cutaneous blood flow responses in one subjects following intradermal injections of placebo, 200 pmol PACAP38 and 200 pmol VIP.

DISCUSSION

MAJOR FINDINGS OF CARBACHOL MIGRAINE MODEL: STUDY I - II Carbachol induced headache in 75% of healthy subjects with a mean peak headache intensity of 1 versus 83% of migraine patients who developed headache with a mean peak headache intensity of 2. Hence, carbachol seems to elicit almost the same headache response in healthy subjects and migraine patients. We did not find that carbachol induced more migraine-like attacks than after placebo. Carbachol also induced a moderate dilatation of cephalic vessels, shown as drop in velocity of about 8% in both healthy subjects and migraine patients, whereas the STA diameter dilated 19% in healthy subjects and 7% in migraine patients.

MECHANISMS OF CARBACHOL INDUCED HEADACHE

Vasodilatation

Acetylcholine and carbachol cause vessel dilatation primarily induced through activation of eNOS (58, 139) and thereby pro-

duction of NO. NO then diffuses into smooth muscle cells, activates guanylyl cyclase and increases cGMP which leads to vasodilatation by decreasing intracellular calcium (140). However, acetylcholine and carbachol induced dilatation might also be mediated via production of prostaglandins (141, 142) as well as endothelium-derived hyperpolarising factor (EDHF) (141, 143, 144). The contribution of these mediators in dilating vessels seems to vary between vessel size (139, 142) and remains to be fully determined.

Glyceryl trinitrate (GTN), a well-known migraine trigger and NO donor (11), easily passes the BBB. It has been suggested that GTN induced migraine-like attacks could be due to cephalic vasodilatation (145), sensitization of perivascular nociceptors (146) and a central effect at second order neurons in the TNC (147, 148). Given that carbachol does not pass the BBB, the current results suggest that headache might be induced by activation of endothelial NO production. However, the GTN study reported median peak headache VRS intensity of 5.5, a decrease in VMCA of 30% (11), and, using a lower dose of GTN, an increase in STA diameter of 37% in migraine patients (149). In comparison, following carbachol in migraine patients a median peak headache VRS intensity of 2, a decrease in VMCA of 8% and an increase in STA diameter of 7% were observed. This suggests that carbachol in the given dose is weaker than GTN in terms of headache induction and cephalic vasodilatation. However, carbachol was used in the maximal tolerable dose, as pilot studies conducted before the main studies revealed that larger doses induced intolerable systemic side effects in healthy subjects (135). Accordingly, a larger dose of carbachol could not be applied. Based on these results we cannot prove or falsify the hypothesis that activation of muscarinic endothelial receptors on cephalic vessels induces sufficient amounts of endothelial NO to induce migraine-like attacks.

Migraine attacks have been shown to be associated with dilatation of cephalic vessels (150, 151). Acetylcholine is quickly degraded by acetylcholine esterase and the distance from nerve ending to endothelial cells has been reported to be more than 500 nm wide (36). However, radio-labelled acetylcholine is able to diffuse, within a few seconds, from the abluminal to the luminal side in concentrations sufficient to induce dilatation shown in rabbit central ear arteries (152). Furthermore, noxious stimulation of rat facial mucosa increases meningeal blood flow, via the trigemino-parasympathetic reflex (25). This was completely blocked by topical dural application of the muscarinic antagonist, atropine (25).

Thus, it is possible, but not yet clarified, whether neuronal acetylcholine or acetylcholine of endothelial origin can activate vascular endothelial cells during spontaneous migraine attacks.

Mast cells

During recent years evidence for mast cell degranulation in migraine pathophysiology has increased (153, 154). The evidence is primarily based on studies showing plasma histamine levels are elevated during migraine attacks in a sub-population of migraine patients (155), histamine induces migraine-like attacks following intravenous infusion (12) and that mast cell degranulation causes activation of meningeal nociceptors in the rat dura mater (156, 157). However, histamine H1 and H2 blockers had no effect in a double-blind placebo controlled study (158) and a mast cell stabilizer also failed as migraine prophylaxis in a double-blind placebo controlled cross-over study (159).

Rat dural mast cells are found in close proximity to AChE immunostained nerve fibres (31). Acetylcholine as well as carbachol induce degranulation of mast cells (31, 73, 160, 161) which can be blocked by muscarinc receptor antagonists (73). Acetylcholine could thus be released and induce mast cell degranulation during spontaneous migraine attacks. Carbachol infused in the present studies might have penetrated dural vessels and induced degranulation of perivascular mast cells that could have contributed to the mild headache.

Sensory activation

Intrabrachial artery infusion of acetylcholine induces moderate to severe pain in the arm (162) and acetylcholine induces a stinging and burning pain when applied to a blister base in healthy subjects (163).

Nicotine applied to the nasal cavity produces a burning and stinging sensation in man and electric impulses are believed to reflect C-fibre and Aδ-fibre activation (164). Furthermore, at low concentrations acetylcholine potentiates capsaicin evoked calcitonin gene related peptide (CGRP) release by nicotinic receptors and inhibits by muscarinic receptors at high concentration in rat trigeminal sensory afferents (165). This is in agreement with Bernadini et al (166) who showed nociceptor excitation via nicotinic receptors, whereas the M2 receptor mediated depression of nociceptive responsiveness in isolated rat skin. Hence, acetylcholine can exert both inhibitory and excitatory actions on sensory afferents. Interestingly, 5-hydroxytryptamine (5-HT) directly inhibits neuronal nicotinic receptors in rat trigeminal ganglion neurons (167). Whether 5-HT1B/1D receptor agonists, which are effective in migraine treatment (168), also have such an effect is currently unknown. Intra-arterial infusion of carbachol in doses similar to the present study induces PPE in rat dura mater (27) mainly mediated via muscarinic receptors. It was also 50% reduced by capsaicin pre-treatment, which depletes sensory afferents. These findings suggest carbachol induced PPE to be caused by both mast cell degranulation and activation of sensory afferents. Thus, nicotinic receptors on sensory afferents can induce activation of nociceptors resulting in pain and might have a role in migraine pathophysiology. Carbachol infused intravenously in study I and II may directly excite C-fibres after penetrating dural vessels explaining the resultant mild headache, but it was not sufficient to induce migraine-like attacks.

MAJOR FINDINGS OF PACAP38 MIGRAINE MODEL: STUDY III The study showed that PACAP38 induced mild to moderate headache in all healthy subjects and most migraine patients. Furthermore, seven migraine patients experienced migraine-like attacks after PACAP38 and none after placebo mostly during the posthospital phase. PACAP38 also induced dilatation of cephalic vessels manifested by a 16% drop in VMCA, whereas the STA diameter dilated 38% in migraine patients.

MECHANISMS OF PACAP38 INDUCED HEADACHE AND MIGRAINE-LIKE ATTACKS

Vasodilatation

In the present study PACAP38 induced cephalic vasodilatation similar to that caused by the parasympathetic neurotransmitter VIP (104). Yet, VIP infusion did not evoke migraine (105) and only mild headache (104). In the VIP study, changes in HR and VMCA returned to baseline 40 min after infusion start, whereas the STA diameter was still increased, though only 10%, 90 min after infusion start (105). In comparison, following PACAP38 infusion both the HR and STA diameter were increased 30% and the VMCA decreased 10%, at 90 min after the infusion start (137). Thus, PACAP38 had a larger duration of action than VIP.

VIP and PACAP38 induced dilatation are endotheliumindependent in some animal studies (169-171). The VPAC1 receptor is primarily responsible for vasodilatation in rat cerebral arteries (101) and has been located to the VSMC in rat cerebral arteries and arterioles (172). Conversely, an endothelial VPAC1 receptor and a VSMC VPAC2 receptor is expressed on porcine cerebral arteries (173), where the dilator response mediated by the endothelial VPAC1 receptor is NOS dependent (173). In humans, PACAP38 induced dilatation is endotheliumindependent in the coronary arteries (171), but endothelium and NOS dependent in pulmonary arteries (174). However, mRNA for the VPAC and PAC1 receptors is detected by reverse transcriptase polymerase chain reaction (RT-PCR) in human cerebral arteries and is not affected by endothelial denudation (99). Thus, the mechanisms responsible for PACAP38 induced dilatation are complex and may have multiple and different pathways depending on species and organ. If PACAP38 induced vasodilatation is primarily NO dependent, it could be argued that liberated NO might induce the headache and migraine-like attacks. This is supported by the fact that the median time to peak headache in our study was 4 h, close to 5.5 h observed after GTN infusion in migraine patients (11).

Following intravenous PACAP38 administration only 0.053% passes the BBB after 5 min via a saturable mechanism in mice (175). PACAP38 infused in the same amount as in our study results in a peak plasma concentration of 37 pM (102). A concentration of 2692 pM PACAP27, equipotent to PACAP38 (5), induces 50% of the maximal relaxation of precontracted human cerebral vessels in vitro (176). This concentration is more than 100,000 times larger than 0.053% of 37 pM (0.02 pM) PACAP38 passing the BBB. Based on these data, it seems unlikely that the MCA dilatation found in our study is primarily caused by PACAP38 penetrating the BBB and activating receptors on the VSMC in the MCA. Yet, the STA dilatation might also involve activation of receptors on the VSMC.

Mast cells

VPAC2 receptors, but not VPAC1, are expressed on human mast cells (177). So far no studies have investigated the expression of PAC1 receptors on mast cells. In human skin PACAP38 and VIP degranulate mast cells and cause histamine release in vitro (95). VIP seems to be the more potent than PACAP38 in degranulating mast cells in vitro (95). Furthermore, VIP releases a quite small proportion (10%) of histamine from human dural mast cells compared to CGRP (32%) (178). Since only a mild headache was reported following VIP (105), dural mast cell degranulation alone is not a likely cause of PACAP38 induced headache or migraine-like attacks in the present study. It could be argued that since PACAP38 has a higher uptake rate into the brain than VIP (179), even though minimal (175), sufficient amounts of PACAP38 might have passed the BBB and degranulated leptomeningeal mast cells (35), contributing to headache and migraine-like attacks.

Peripheral sensory activation

Stimulation of both VPAC and PAC1 receptors elevates cAMP (180), but PACAP38 has been reported to stimulate adenylate cyclase activity at least 1000 times more than VIP in cultured neural cells (181). CGRP and cilostazol increase cAMP and cause headache in healthy subjects (182, 183). Animal models in both rats (184) and guinea pigs (185) have shown that trigeminal neurons can be sensitized through elevation of cAMP. However, it has not yet been demonstrated if VPAC or PAC1 receptors mediate sensitization of trigeminal nociceptors.

Interestingly, VIP and PACAP38 potentiate nicotinic evoked currents in chick embryonic parasympathetic neurons by increasing cAMP (186, 187), and PACAP38 is more efficient in potentiating the acetylcholine sensitivity (186). It would be interesting to explore if PACAP38 might also potentiate nicotinic evoked currents in trigeminal nociceptors. This would point to a synergetic effect between PACAP38 and acetylcholine that might occur during efferent outflow from parasympathetic perivascular nerve endings.

Stimulation of the superior sagital sinus causes a 2.6-fold increase in PACAP plasma concentrations in the external jugular vein in cats (188), but whether this is caused by release from trigeminal or parasympathetic fibres remains to be determined. Since parasympathetic and trigeminal fibres are closely related in the perivascular space (31), it is possible that PACAP released from either system could lead to modulation of trigeminal sensory neurons.

Central sensory activation

PACAP-immunoreactivity is present in the human TNC (89). PACAP-immunoreactivity was also found in cell bodies of the brain stem locus coeruleus (189) that send projections to the TNC (190) and is reported to be activated during spontaneous migraine attacks (191). Animal models have proposed that PACAP might have a role in central pain transmission (103). Capsaicin elevates PACAP in rat cerebrospinal fluid in vivo (192) suggesting PACAP is released from activated C-fibres in the spinal cord. In PACAP-/- gene knockout mice inflammatory pain disappears (193), and PACAP promotes the functional coupling of neuronal NO-synthase to N-methyl D-aspartate receptors. This leads to NO production in superficial layers of the dorsal horn in the spinal cord (193) and late-onset, transcriptional- and activity-dependent central sensitization (194). Interestingly, PAC1-/- knock out mice have a decreased response in nociceptive behaviour after a formalin test (195), which is a model of inflammatory nociception. Furthermore, the PAC1 receptor antagonist, PACAP 6-38, effectively attenuated nociception in inflammatory pain models in rats (196) and mice (197) after intrathecal administration, suggesting that PAC1 receptor is pronociceptive at the central level of the spinal cord. A central mechanism of PACAP38 infusion in the

present study might be relevant, since the peak headache occurred 4 h after PACAP38 infusion. However, given the moderate amount of PACAP38 that passes the BBB (175), it is unknown if this would have any direct physiological effects in the central pain transmission.

LIMITATIONS OF THE HUMAN MIGRAINE MODEL: STUDY I - III Adverse events following carbachol and PACAP38 infusion may to some extent have compromised blindness of the studies, but adverse events were caused by the physiologic response to carbachol and PACAP38 and could not have been avoided. The present double-blind approach was the best possible way of coping with methodological errors. Though carbachol induces changes by affinity of the same receptors (198) a direct comparison between carbachol and acetylcholine is difficult and the effects of carbachol can be less NO dependent than acetylcholine (199). However, it is practically impossible to administer acetylcholine in a human headache model, and carbachol is the best agonist to mimic the effects of acetylcholine. The high placebo response of migraine-like attacks (33%) could influence the results of study II. It is difficult to interpret this unusual placebo response, but it is possible that the migraine patients were more sensitive to the stress of participating in the study, compared to migraine patients in previous studies. This emphasizes the importance of conducting human experimental headache studies in a placebo-controlled double-blind crossover design. The use of rescue medication in the studies might blur the true headache responses to the experimental drugs, but for ethical reasons patients in experimental provocation studies cannot be denied treatment of the induced attacks. Secondary vasodilatation might have occurred during the post-hospital phase following PACAP38 infusion, which could be detected via transcranial Doppler, and would be important to record. However, we did not find it practically possible to record vascular effects for several hours in study III.

MAJOR FINDINGS IN THE CUTANEOUS MODEL OF ACUTE PAIN: STUDY IV

VIP and PACAP38 evoked more cutaneous pain, central sensitization, neurogenic inflammation and mast cell degranulation when compared to placebo. VIP was more potent in inducing neurogenic inflammation and mast cell degranulation than PACAP38. VPAC1 and/or VPAC2 receptor activation seem to induce these phenomena, as additional activation of the PAC1 receptor by PACAP38 apparently had no effect on pain or other responses. The pain area was also larger after VIP and PACAP38 than placebo, indicating that the peptides activated mechano-insensitive silent nociceptors which have larger receptive fields than mechano-responsive nociceptors (200).

Pain in relation to the human migraine model

In the present study 200 pmol of VIP or PACAP38 injected at a single point into the dermis induced short lasting pain. This was caused by a high local concentration of peptides in the dermis, which cannot be expected to be reached when 200 pmol/kg VIP or PACAP38 are intravenously infused. In human experimental migraine studies, an experimental drug has never induced pain elsewhere than the head (12, 13, 105, 182, 201-204). Thus, cephalic vessels are either innervated by nociceptors with different

and more sensitive properties than in other organ compartments, or headache is caused by other mechanisms.

Central sensitization

The ability for VIP and PACAP38 to induce central sensitization, shown as punctuate hyperalgesia, is interesting as central sensitization is believed to occur during spontaneous migraine attacks (134).

So far, central sensitization during spontaneous migraine attacks has mainly been suggested by headache features and experimental signs of allodynia (134, 205, 206). Punctuate hyperalgesia would not be reported by migraine patients, unless specifically tested in an experimental setting, whereas allodynia is revealed as pain during movement of the head, when coughing or touch of the skin. In fact, 58% of the migraine patients complained of headache aggravation by movement after PACAP38 infusion (137). However, since VIP also induced central sensitization, we cannot translate this finding as a necessary mechanism for the induction of PACAP38 induced migraine. Furthermore, central sensitization during migraine attacks takes hours to develop (134) and might be generated by other mechanisms than in the human skin.

Neurogenic inflammation

Neurogenic inflammation in the skin is caused by activation of nociceptors, which via an axon reflex release substance P (SP) and CGRP leading to increased skin blood flow and flare in the skin (207). In the cutaneous study VIP increased skin blood flow more than PACAP38, which is in agreement with a previous study (208). Intradermal histamine injection induces increased skin blood flow and flare area (209). Given that VIP induces greater histamine release than PACAP38 (95), histamine might contribute to a larger increase in skin blood flow and flare reaction.

The neurogenic inflammation hypothesis in migraine pathophysiology is based on animal studies and states that peripheral endings of nociceptors, innervating meningeal vessels, release SP leading to PPE and CGRP leading to vasodilatation (210-212). This may further promote and sustain activation and sensitization of meningeal nociceptors during migraine attacks (6). Intradermal injections of capsaicin (126) and serotonin (213) both induce flare (i.e. neurogenic inflammation) in human skin and activate and sensitize rat dural nociceptors (6, 157). However, prostaglandin D2 injections in human skin induce flare (214), but is largely ineffective in activating and sensitizing rat dural nociceptors (157), whereas prostaglandin I2 injections in human skin cannot induce flare (215), but robustly activates and sensitizes rat dural nociceptors (157). Furthermore, CGRP intradermal injection induces no pain or flare (215, 216) and fails to sensitize or activate rat dural nociceptors (217), but is known to induce headache and migraine-like attacks (13). Electrical stimulation of human skin induces only flare and no PPE, in contrast to rat skin where both flare and PPE are induced (218). Retinal PPE is seen in rats following high-intensity electrical stimulation of the trigeminal ganglion but not in humans during migraine attacks (219). This may be the reason why SP antagonists fail in the treatment of migraine attacks (220), although they potently inhibit electrically evoked PPE in rat dura mater (221). In summary, previous data, and the present study showing VIP to be more potent than PACAP38 in inducing skin neurogenic inflammation, questions the

importance of neurogenic inflammation in migraine and emphasize the importance of reservations when translating results from different organs and species into migraine pathophysiology.

Mast cells

Intradermal injection of VIP induces histamine release in human skin in vivo measured with microdialysis technique (222) and produces a wheal and flare reaction within a few minutes of similar degree as in the present study (215, 223, 224). Topical capsaicin pre-treatment, known to inhibit neurogenic inflammation by depletion of nociceptors, robustly inhibits VIP induced skin flare, but does not affect the wheal reaction (225) which most likely is caused by mast cell degranulation. Even though it is unknown why PACAP38 shows lower efficacy in histamine release from mast cells (95) it is in agreement with the

histamine release from mast cells (95), it is in agreement with the larger wheal area following VIP injection compared to PACAP38 in the present study. Though it is unknown, it would be very surprising, given that VIP induced a significant larger wheal area, if PACAP38 infusion induced a larger mast cell degranulation than VIP in the dura mater. Consequently, the present study does not support mast cell degranulation to have any major role in PACAP38 induced migraine-like attacks.

LIMITATIONS OF THE CUTANEOUS MODEL OF ACUTE PAIN: STUDY IV

The flare and wheal responses following VIP and PACAP38 injections compared to placebo might have compromised blindness of the study. However, the subjects kept their eyes closed during testing, and we experienced that they did not examine their arm closely in between the testing. A more intense and robust pain induction following injection would have been desirable. However, 200 pmol is a large dose for intradermal injections, so the pain reported probably reflects that the peptides do not activate cutaneous nociceptors very effectively.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The human migraine model provides important information on the role of perivascular signalling molecules in head pain and migraine. The present thesis demonstrated that infusion of the cholinomimetic agent carbachol infusion was not a good model for migraine provocation as the maximal tolerable carbachol dose had minimal vascular and headache inducing effects, probably due to the limited amounts of carbachol reaching cephalic vessels. Therefore, we cannot exclude that perivascular release of acetylcholine may have a pronociceptive effect during spontaneous migraine attacks. The role of acetylcholine in migraine pathophysiology might be investigated in experimental animal models, where the effects of topically applied cholinomimetic drugs can be studied. The muscarinic receptors have both excitatory and inhibitory effects depending on their subtype (165, 166). Specific muscarinic receptor subtype antagonists and agonist developed for clinical human use in the future would certainly be of interest to test as anti-migraine compounds.

PACAP38 infusion provoked migraine-like attacks in migraine patients and thereby revealed a new migraine pathway. Several mechanisms are possible for the induction of migraine. The infusion of PACAP38 induced dilatation of cephalic vessels lasted longer than 90 min and seemed to have longer duration in comparison with VIP infusion in healthy subjects (104) and MO patients (105). However, VIP and PACAP38 need to be studied in a head to head comparison, with several hours of monitoring, to show that PACAP38 has prolonged vascular effects compared to VIP. PACAP induced cephalic vasodilatation might be NO dependent. Thus, NO liberation is a possible cause of migraine-like attacks following PACAP38 infusion. It would be very interesting to test if NOS inhibitors could block PACAP38 induced vasodilatation and migraine-like attacks.

The cutaneous pain model did not reveal why PACAP38 and VIP are markedly different in migraine induction. Interestingly, the cutaneous study results question if mast cell degranulation and neurogenic inflammation are relevant causes of PACAP38 induced migraine. Yet, reservations are important as it is unknown to what extend the data obtained from the cutaneous compartment can be extrapolated to perivascular cephalic structures. The mast cell mechanism should still be investigated in a human model. Measuring histamine plasma levels following PACAP38 and VIP infusion would be of interest to elucidate, preferably from both the external jugular and cubital vein, to differentiate between systemic and cephalic mast cell degranulation. Pre-treatment with histamine receptor antagonists might also reveal if mast cell degranulation is a causative factor for migraine development following PACAP38 infusion.

The cutaneous model showed that VIP and PACAP38 induced a mild acute pain of similar intensity but in the migraine model the headache peaked several hours latter. Thus, for the development of headache a cascade of event is likely taking place. Future experiments should attempt to elucidate these cascade mechanisms. Human experiments during delayed migraine attacks might detect the development of central sensitization using quantitative sensory testing. Experimental animal models would be useful in elucidating peripheral and central sensory mechanisms by using single cell recordings and local application to the dura mater (156) or to brain stem nuclei (226). Also, synergetic mechanisms between PACAP and other perivascular signalling molecules, such as acetylcholine, could provide valuable information on nociceptive mechanisms relevant for migraine. Based on the experimental findings in the present thesis we suggest PAC1 receptor antagonism as a future target for anti-migraine treatment. At the moment there are no PAC1 agonists or antagonists available for human use, but experimental animal models could test nociceptive effects of specific receptor activation. Since vasodilatation seems to be mediated by VPAC1 receptor activation, PAC1 antagonists might be without vascular side ef-

activation, PACI antagonists might be without vascular side effects. Furthermore, if PACAP release in the perivascular space is a trigger of spontaneous migraine attacks, PAC1 receptor antagonist could also be effective as migraine prophylactic treatment.

In conclusion, the present thesis contributes to our knowledge on how acetylcholine and PACAP might be involved in migraine pathophysiology and provides data that could lead to new antimigraine treatment strategies.

SUMMARY

The parasympathetic signalling molecules acetylcholine, pituitary adenylate cyclase activating peptide-38 (PACAP38) and vasoactive intestinal peptide (VIP) may be released from parasympathetic fibres and activate sensory nerve fibres during migraine attacks. Recently, it was shown that VIP does not induce migraine-like attacks in migraine patients. Interestingly, PACAP38 activates the same VPAC receptors as VIP, but also specifically activates the PAC1 receptor.

The present thesis includes four double-blind placebo-controlled crossover studies aimed to explore the role of acetylcholine, PACAP and VIP in migraine and head pain. In study I-III we investigated acetylcholine, via the analogue carbachol, and PACAP38 in a human model of migraine. In study IV we studied if PACAP38 and VIP might induce central sensitization, neurogenic inflammation and mast cell degranulation in a cutaneous model of acute pain.

Study I-II showed that carbachol induced short lasting mild headache and moderate cephalic vasodilatation in both healthy volunteers and migraine patients, but did not induce migraine-like attacks. In study III PACAP38 induced headache in healthy subjects and delayed migraine-like attacks in migraine patients as well as sustained dilatation of cephalic vessels. In study IV VIP and PACAP38 evoked skin pain, central sensitization, neurogenic inflammation and mast cell degranulation, but VIP showed to be more potent than PACAP38 in inducing neurogenic inflammation and mast cell degranulation.

In conclusion, we found that carbachol infusion was not a good model for experimental migraine provocation, probably because the maximal dose was insufficient to produce enough nitric oxide to trigger migraine. PACAP38 infusion is a new pathway for migraine induction and the results from study IV suggests that neurogenic inflammation and mast cell degranulation are unlikely to cause PACAP38 induced migraine. The present thesis contributes to our knowledge on migraine pathophysiology and suggests PAC1 receptor antagonism as a new target for migraine treatment.

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ABBREVIATIONS

ACA	anterior cerebral artery
AChE	acetylcholine-esterase
AUC	area under the curve
BA	basilar artery
BBB	blood-brain-barrier
cAMP	cyclic adenosine monophosphate
CBF	cerebral blood flow
CGRP	calcitonin gene-related peptide
ChAT	choline acetyltransferase
eNOS	endothelial NO synthase
GTN	glyceryl trinitrate
HR	heart rate
IHS	International Headache Society
MAP	mean arterial pressure
MCA	middle cerebral artery
MO	migraine without aura
MPQ	McGill pain questionnaire
NO	nitric oxide
OM	orbito-meatal
PACAP	pituitary adenylate cyclase activating peptide
PetCO2	end-tidal partial pressure CO2
PLC	phospholipase C
PPE	plasma protein extravasation
RA	radial artery
rCBFMCA	regional cerebral blood flow in the MCA area
SP	substance P
SPECT	single photon emission computer tomography
SPG	sphenopalatine ganglion
SSN	superior salivatory nucleus
STA	superficial temporal artery
TCD	transcranial Doppler ultrasonography
TNC	trigeminal nucleus caudalis
VAS	visual analogue scale
VFH	von Frey hair
VIP	vasoactive intestinal peptide
VMCA	velocity of the middle cerebral artery
VRS	verbal rating scale
VSMC	vascular smooth muscle cell
5-HT	5-hydroxytryptamine

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