Increase of meningeal blood flow after electrical stimulation of rat dura mater encephali: mediation by calcitonin gene-related peptide

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1 The dura mater encephali of the rat was exposed and the blood flow around branches of the medial meningeal artery was monitored with a laser Doppler flowmeter. Changes in the meningeal blood flow (MBF) following electrical stimulation of the dura mater at a parasagittal site were registered. The effects of human calcitonin gene-related peptide (h-αCGRP) and the CGRP antagonist (h-αCGRP₈₋₃₇) on the MBF were tested.

2 Electrical stimulation with rectangular pulses of 0.5 ms, 10–20 V, 5–10 Hz and a duration of 30 s caused an increase of the MBF in 14 out of 16 rats tested. The increases were dependent on stimulus strength and frequency.

3 The increase in MBF was inhibited in a dose-dependent manner by topical application of 0.1 ml of h-αCGRP₈₋₃₇ at concentrations of 10⁻⁷–10⁻⁵ M. The highest dose abolished the increase in MBF.

4 Topical administration of 0.1 ml of h-αCGRP at a concentration of 10⁻⁴ M increased the basal MBF by 15% on average.

5 It is suggested that the increase in MBF following electrical stimulation of the dura mater is mediated by the release of CGRP. The contribution of the dural afferent and sympathetic and parasympathetic efferent nerve fibres to this response are discussed.

Keywords: Neurogenic inflammation; dura mater encephali; meningeal afferents; neuropeptide release; CGRP antagonist; blood flowmetry

Introduction

Neurogenic inflammatory responses of meningeal tissues, e.g. vasodilatation and plasma extravasation in the dura mater, have been proposed as an essential mechanism for migraine pain and other headaches (Moskowitz, 1984; 1993; Hardebo, 1990). In the rat a neurogenic inflammation of the dura mater was provoked by stimulation of trigeminal afferents, indicated by symptoms like plasma extravasation (Markowitz *et al.*, 1987), aggregation and adhesion of platelets, and activation of mast cells (Dimitriadou *et al.*, 1991; 1992). These responses are likely to be mediated by neuropeptides released from the peripheral terminals of fine trigeminal afferent fibres.

Histochemical studies by von Düring *et al.* (1990) and Keller & Marfurt (1991) as well as our recent investigation (Messlinger *et al.*, 1993) revealed that the dura mater encephali of the rat is densely innervated by both substance P and calcitonin gene-related peptide (CGRP) immunoreactive nerve fibres of small diameter suggesting the presence of these neuropeptides within distinct classes of dural afferents. Whereas substance P (as well as neurokinin A) seems to mediate mainly plasma extravasation that can be inhibited by the NK₁ receptor antagonist, RP 67580 (Moussaoui *et al.*, 1993; Shepherd *et al.*, 1993), it is suggested that CGRP contributes little to this response (Markowitz *et al.*, 1987). On the other hand, CGRP is well-known as a powerful vasodilator in various tissues including cerebral cortex (Brain *et al.*, 1985; McCulloch *et al.*, 1986; Andersson, 1989; Edwards *et al.*, 1991). In the dura mater a vasodilator action of CGRP has not so far been examined. Many of the CGRP immunoreactive fibres follow the course of the medial meningeal artery (MMA) up to the superior sagittal sinus, ramify

in the parietal area of the dura mater and terminate both at dural vessels and within dural connective tissue (Messlinger *et al.*, 1993). Release of CGRP from these afferent terminals would be expected to dilate dural vessels and increase meningeal blood flow.

The aim of this study was to examine whether activation of meningeal afferents by electrical stimulation of the dura mater increases the meningeal blood flow, and whether these changes can be modified by the CGRP antagonist, h-CGRP₈₋₃₇, indicating an involvement of CGRP. Briefly, our measurements provide convincing evidence for a vasodilator action of CGRP released in meningeal structures after electrical stimulation of the dura mater.

Methods

Anaesthesia and general preparation

The study was performed on 16 Wistar rats of either sex (190–480 g). They were anaesthetized by an initial dose of 100–120 mg kg⁻¹ thiopentone, (Trapanal) i.p., followed by additional doses of 2–25 mg kg⁻¹ thiopentone i.p. when required. Depth of anaesthesia was routinely judged by observing the immobility to noxious stimuli and by monitoring the arterial blood pressure of the animal. The trachea was cannulated, and spontaneous respiration was assisted by blowing a gentle jet of oxygen toward the opening of the tracheal cannula. A catheter was placed into the right femoral vein for the i.v. infusion of solutions when necessary. Arterial blood pressure was continuously recorded with a pressure transducer via a catheter in the right femoral artery and maintained above 100 mmHg (systolic) by i.v. infusion of 4% Ficoll 70 (Serva, Heidelberg) and 5% glucose solu-

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tions. The animal's body temperature was maintained at 37.0–37.5°C with a thermostatically regulated heating plate.

Preparation for stimulation and recording

The animal's head was fixed in a stereotaxic frame. The skin covering the dorsal side of the head was divided by a sagittal section and pulled aside. Periosteum and the dorsal part of the masseter muscle were removed. The skull was carefully opened by means of an electric drill while being cooled with Tyrode solution (4°C). Two openings were formed for exposing the dura mater (Figure 1): one parasagittal slit of about 2 x 6 mm for electrical stimulation, and one rectangular window of about 5 x 6 mm in the parietal bone exposing the

Figure 1 Experimental set-up for electrical stimulation of the rat dura mater by electrodes (Stim) oriented in parallel to the sagittal suture (SS) and for recording of the meningeal blood flow (Rec) around the middle meningeal artery (MMA) with a needle probe (tip diameter 1.0 mm) of a laser Doppler flowmeter. CS coronal suture, MCA branches of the medial cerebral artery visible through the dura.

distal branches of the medial meningeal artery (MMA). A needle type of probe of a laser Doppler flowmeter was placed over a branch of the MMA without touching it. The parietal opening was covered with pieces of gauze soaked with Tyrode solution. A pair of parallel stimulating electrodes (wire diameter 0.2 mm, distance 1 mm) with a length of 4 mm was lowered onto the dural surface of the parasagittal opening, so that the wires touched the dural surface along their whole length parallel to the sagittal suture. The opening was filled with a cotton pad soaked with liquid paraffin.

Electrical stimulation and recording of blood flow

The dura was electrically stimulated by an isolated stimulator (HI-Med) with rectangular pulses of a constant length of 0.5 ms. Stimulus strength and frequency were varied from 5–20 V and 2–20 Hz, respectively, and were optimized at the beginning of each experiment to elicit maximal elevations of blood flow without changing the blood pressure (Figure 2). Blood flow was measured by a laser Doppler flowmeter (Moor Instruments, MBF3D; time constant 1.0 s) and recorded on-line by a penwriter (Rikadenki). Each test period was preceded by at least 4 control stimulations eliciting stable responses at intervals of 5 min. Then the test substance (on a gauze pad soaked with 0.1 ml solution of agents) was applied topically to the exposed dural surface. Two minutes later the first test response was elicited followed by further stimulations at intervals of 5 min.

Drug administration

Human calcitonin gene-related peptide (h-αCGRP, Bachem Chemicals) was administered at a concentration of 10^{-4} M, the CGRP antagonist h-αCGRP₈₋₃₇ (Bachem) at concentrations of 10^{-7} , 10^{-6} and 10^{-5} M dissolved in Tyrode solution. The chemicals were kept frozen in a stock solution (10^{-4} M) and diluted immediately before use.

Evaluation of data

Changes of blood flow after electrical stimulation were assessed by measuring the area under the evoked response from the baseline before stimulation to the point where the curve returned to the baseline (see Figure 2) using morphometric software (HISTOL, List electronics). A constant

Figure 2 Increases in meningeal blood flow induced by electrical stimulation with pulses of 0.5 ms length for 30 s. (a) Variation of the stimulus strength (frequency kept constant at 10 Hz). The blood flow response was maximal at 17.5 V. (b) Variation of the stimulus frequency (strength constant at 15 V). The blood flow was optimal at 5–10 Hz. Higher frequencies caused big fluctuations of the blood flow and slightly lowered the systemic blood pressure (BP).

flow expressed by a horizontal baseline was a prerequisite for starting the test period. The increases in blood flow were expressed as relative values compared to the last control area (= 100%) immediately before the test sequence (see Figure 3). Comparison of the blood flow responses was made with one-way ANOVA and Fisher's least significance test.

Results

Increase of blood flow after electrical stimulation

In 14 of 16 experiments, electrical stimulation of the dura mater encephali via surface electrodes (see Figure 1) increased the blood flow that was measured at a parietal site over a branch of the medial meningeal artery. This increase was dependent on the stimulus strength and the frequency. Both these parameters were systematically varied in 6 experiments. Figure 2a shows an example where the stimulus strength was varied from 5 to 20 V. The blood flow increase was maximal at a stimulus strength of 17.5 V. As in this experiment, the maximum response was obtained between 10–20 V in all experiments. In Figure 2b the changes of blood flow are shown when the frequency of the stimulation was varied. A maximal effect was observed with stimulation frequencies of between 5 and 10 Hz. Higher frequencies caused a fluctuating response and changes in the systemic blood pressure. In view of these results, all further experiments were carried out with stimulus frequencies of 5 or 10 Hz. The duration of the blood flow response achieved by these stimulation parameters ranged from 1–2 min.

In two experiments the blood flow decreased during stimulation. This decrease could be abolished by local app-

lication of phentolamine at a concentration of 3 mM showing that it was due to an α -sympathetic activation. These two experiments were excluded from further analysis.

Inhibitory effect of h-CGRP₈₋₃₇ on blood flow increase

In 5 experiments Tyrode solution, topically administered on the dura mater as a control, did not change the evoked blood flow increase (Figure 3a). The effect of 100 μ l of the CGRP antagonist, h-CGRP₈₋₃₇ at concentrations of 10^{-7} up to 10^{-5} M, administered in the same way, was examined in 10 experiments. In all these experiments h-CGRP₈₋₃₇ diminished the electrically evoked blood flow response measured around the MMA. In the experiment depicted in Figure 3 the response was reduced to about 30% of the control responses (at 2, 7 and 12 min) after the application of h-CGRP₈₋₃₇ (10^{-6} M), and it gradually returned toward the control response size.

The reduction of the electrically evoked blood flow response depended on the dose of h-CGRP₈₋₃₇ applied (Figure 4). H-CGRP₈₋₃₇ at a concentration of 10^{-7} M initially reduced the response to about 60% of its control size on average ($n = 4$ experiments). The inhibitory effect was short-lasting, i.e., it was significant only 2 min after administration of h-CGRP₈₋₃₇. The evoked increase of MBF recovered (without removing the antagonist) within 17 min. The higher concentration of 10^{-6} M caused an initial reduction of 75% on average ($n = 4$). The effect was long-lasting, and recovery was incomplete. The highest dose of h-CGRP₈₋₃₇ (10^{-5} M) totally abolished the blood flow response; the response showed no recovery within the observation time of 40 min.

Isolation of the dura mater

Since the blood flow measurement in this study may include pial and cortical flow in addition to the dural flow, at the end of 4 experiments the dura mater was incised and a thin sheet of black polyethylene was placed under the dura underlying the probe of the Doppler flowmeter to exclude the contribution of the blood flow beneath. In two experiments the electrically evoked increase of MBF did not significantly change after isolation of the dura (Figure 5). In one of these experiments the basal blood flow decreased after this manipulation, in the other experiment it increased accompanied by a bleeding from the lesioned dura mater. In two other experiments the evoked blood flow response decreased

Figure 3 Specimen records of the blood flow responses to electrical stimuli before and at the indicated times after topical administration of Tyrode solution (vehicle) and h-CGRP₈₋₃₇. (a) Tyrode solution did not significantly change the evoked increases of blood flow. In this trial the responses slightly increased. (b) Inhibition of the electrically evoked blood flow responses by h-CGRP₈₋₃₇. In this trial also the basal blood flow was slightly decreased after application of the antagonist, as can be seen in the original records (upper diagram). The lower diagram shows the complete set of blood flow responses of the same trial, expressed as relative values to the control response recorded immediately before the h-CGRP₈₋₃₇ application.

Figure 4 Effects of various concentrations of h-CGRP₈₋₃₇ (10^{-7} M, $n = 4$, $\circ$; 10^{-6} M, $n = 4$, $\bullet$; 10^{-5} M, $n = 4$, $\blacktriangle$) on the blood flow responses evoked by electrical stimulation of the dura mater, compared to the vehicle (Tyrode solution, $n = 5$, $\circ$). Values are mean $\pm$ s.e.mean. Values after administration of h-CGRP₈₋₃₇ were tested for significant differences (* $P < 0.05$; ** $P < 0.01$) versus values after Tyrode solution (one way ANOVA and Fisher's least significance test).

Figure 5 Evoked blood flow responses (bars below responses represent stimulation with 20 V and 10 Hz for 30 s) before and after isolation of the dura mater by inserting a black plastic sheet between dura and cortical surface.

to 36% and 49% respectively, of the previous average value, while the basal blood flow decreased in the first and increased in the latter case.

At the end of two experiments the MMA was selectively coagulated at a proximal site (at the lateral border of the recording window). The basal blood flow measured around the MMA decreased, in one case to nearly zero. In contrast, the basal cortical flow was not significantly changed by coagulation of the MMA when the probe of the Doppler flowmeter was positioned over a branch of the medial cerebral artery.

Increase of blood flow after h-CGRP

In 6 experiments human calcitonin gene-related peptide (100 μ l) at a concentration of 10^{-4} M was administered topically on the dura mater (recording site). In all experiments it increased the basal blood flow (without electrical stimulation), an effect lasting for 4 to 16 min (Figure 6). The peak increase was 15% on average. Tyrode solution as a control did not change the blood flow.

Discussion

The present paper shows that electrical stimulation of the dura mater encephali with stimuli that are able to activate thin myelinated and unmyelinated nerve fibres increased the meningeal blood flow in the rat. This evoked increase was inhibited by the CGRP antagonist, h-CGRP₈₋₃₇, providing evidence that it was mediated by CGRP. Human CGRP itself caused an increase of the basal blood flow without electrical stimulation. Since the dura mater encephali is densely innervated by CGRP immunoreactive nerve fibres (Keller & Marfurt, 1991; Messlinger *et al.*, 1993), it is likely that CGRP is released from these nerve endings when they are activated by electrical stimulation. The same stimuli probably also release substance P (Edvinsson *et al.*, 1983) that is stored in a smaller proportion of dural nerve fibres (von Düring *et al.*, 1990; Messlinger *et al.*, 1993) where it may largely be co-localized with CGRP (O'Connor & van der Kooy, 1988), possibly in the same vesicles (Gulbenkian *et al.*, 1986). Substance P has been shown in different species (man, guinea-pig, cat) to act as a powerful vasodilator on isolated intracranial arteries including the middle meningeal artery *in vitro* (Jansen *et al.*, 1991; 1992) and on cerebral arterioles of the cat *in situ* (Jansen *et al.*, 1991). In our preparation, however, recent experiments have revealed that meningeal blood flow is not significantly changed by topical administration of substance P and the substance P analogue septide at concentrations of up to 10^{-3} M (unpublished results). These results may indicate different mechanisms of the two vasodilator neuropeptides: CGRP may act directly on the smooth muscle cells of arterial vessels, whereas the vasodilator effect of substance P may require the participation of the endothelium (Enokibori *et al.*, 1994) that is less

Figure 6 (a) Increase of meningeal blood flow (Flow) after topical administration of 100 μ l h-CGRP at a concentration of 10^{-4} M. The brief decrease in blood flow immediately after the h-CGRP application was not seen in all experiments. The systemic blood pressure (BP) remained practically constant. (b) Administration of Tyrode solution (vehicle) in the same experiment (dripped onto the cotton pad) did not change the blood flow. The transient fluctuation of the blood flow is probably an artefact because the drop of vehicle briefly alters the amount of light reflected to the sensor.

accessible to topically applied peptides. Additionally, species-specific differences in the effectiveness of substance P may contribute to these discrepancies.

Sympathetic and parasympathetic contribution to blood flow response

It may be argued that electrical stimulation of the dural surface also activates other than afferent fibres, since the dura mater encephali of the rat is richly innervated by sympathetic nerve fibres (Keller *et al.*, 1989) and shows additionally some cholinergic fibres indicative of a parasympathetic innervation (Amenta *et al.*, 1980). Stimulation of the sympathetic nerves would probably decrease the dural blood flow by releasing catecholamines which would cause constriction of blood vessels. In fact, in some of our experiments a blood flow decrease was seen under stimulation. These phenomena may be interpreted by assuming opposite effects on the blood flow caused by vasoconstrictor catecholamines and vasodilator neuropeptides. It is likely that α -adrenoceptors were involved in the decreasing blood flow responses, since these effects could be abolished by phenolamine. It is further possible that in all other experiments

vasoconstrictor effects were present but hidden by the dominant blood flow increase. Assuming that h-CGRP₈₋₃₇ does not interfere with the catecholaminergic system, these effects probably did not alter our measurements that were based on the comparison before and after application of the antagonist.

Stimulation of the postganglionic parasympathetic fibres originating in the sphenopalatine ganglion has been shown to increase cortical blood flow (Suzuki *et al.*, 1990b). It was suggested that this increase was mediated by vasoactive intestinal polypeptide (VIP) which is co-localized in cholinergic nerve fibres. Since the dura mater also shows a sparse VIP-immunopositive innervation (Keller & Marfurt, 1991), it is possible that a small part of the increase of blood flow that persisted after the application of h-CGRP₈₋₃₇ was induced by VIP.

Selectivity of stimulation

Stimulation of the dural surface was used to activate selectively afferents of the dura mater. We know from immunohistochemical studies (Messlinger *et al.*, 1993) that many CGRP-immunoreactive nerve fibres which originate from dural nerves that accompany the MMA are likely to cross the stimulation site. On the other hand, it is unavoidable that at this site afferents are activated that innervate other than dural structures, e.g. the sagittal sinus, and these nerve fibres may have collaterals along the pial venous vessels extending down to the cortex. Electrical stimulation of the superior sagittal sinus of the cat has been shown to produce increases in cerebral blood flow (Lambert *et al.*, 1988). Thus activation of deeper structures, especially nerve fibres of pial vessels, cannot be excluded by our method. Other investigators stimulated the trigeminal ganglion to activate meningeal afferents antidromically and elicit neurogenically mediated plasma extravasation from dural blood vessels (Markowitz *et al.*, 1987; Dimitriadou *et al.*, 1991; Shephard *et al.*, 1993). Since the trigeminal ganglion of different mammals gives rise to a dense CGRP peptidergic innervation of cerebral vessels (McCulloch *et al.*, 1986; Tsai *et al.*, 1988; Nozaki *et al.*, 1990) by pathways that do not project to the dura mater, e.g. through the nasociliary nerve (Suzuki *et al.*, 1989), stimulation of the trigeminal ganglion may be less appropriate for a selective activation of dural afferents than the method used in this study.

Selectivity of measurement

Suzuki *et al.* (1990a) showed that stimulation of the nasociliary nerve increases the cortical blood flow in the cat. In a recent study this increase could be inhibited by h-CGRP₈₋₃₇ demonstrating that CGRP contributes to this response (Goadsby, 1993). Our study suggests that a similar

mechanism exists in the dura mater. Isolation of the dura mater as well as coagulation of the MMA showed that the blood flow registered in our experiments consisted mainly of dural blood flow.

Relevance of CGRP-dependent increase of meningeal blood flow during migraine attack

Several studies during the last decade using different methods have confirmed that migraine (migraine with aura and less consistently also migraine without aura) is accompanied by significant changes of the cerebral blood flow (see Friberg, 1991; Olesen, 1991 for recent overviews). Precise measurements with a sufficient time resolution showed that hypoperfusion is changed into hyperperfusion during the headache phase of migraine (Olesen *et al.*, 1990), probably due to the dilatation of large cerebral arteries (Friberg *et al.*, 1991). Thus both the migraine headache and the increased blood flow may depend on the same mechanisms. Results from several studies point to a neurogenic mechanism involved in migraine pain. A neurogenic inflammation of the meninges was proposed by Moskowitz and coworkers for the development of migraine headache (Moskowitz, 1984; 1993). This hypothesis was strengthened by several *in vitro* and *in vivo* experiments in which they measured plasma exudation and pathological changes of blood vessels and mast cells in the dura mater following antidromic stimulation of trigeminal afferents in the rat (Markowitz *et al.*, 1987; Dimitriadou *et al.*, 1991; 1992). Second, during migraine attacks increased levels of CGRP were found in the jugular vein of the affected side (Goadsby *et al.*, 1990). In analogy to this, an outflow of CGRP in the jugular vein of the cat (Goadsby *et al.*, 1988) and in the superior sagittal sinus of the rat (Buzzi *et al.*, 1991) could be measured after electrical stimulation of the trigeminal ganglion. These experiments suggest that the increase of cerebral blood flow during migraine attacks is at least partially due to a vasodilator effect of CGRP probably released from trigeminal fibres that innervate intracranial vessels. The results of the present study are consistent with this hypothesis. We conclude that the release and vasodilator action of CGRP following afferent stimulation take place in the dura mater as well as in pial structures increasing both cortical and meningeal blood flow. These processes may also occur in man during migraine headache.

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