Calcitonin Gene-Related Peptide and Migraine:

Implications for Therapy

Udayasankar Arulmani

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Calcitonine gen-gerelateerde peptide en migraine:

Implicaties voor therapie

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Udayasankar Arulmani

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Promotor: Prof.dr. P.R. Saxena

Overige leden: Prof.dr. C.M. Villalón
                Prof.dr. D.J. Duncker
                Prof.dr. C.I. de Zeeuw

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CHAPTER 1

Introduction

1   Introduction

1.1   Historical Perspective

It is clearly evident from the literature that headache has troubled mankind from the dawn of civilization (Rapoport & Edmeads, 2000). A variety of methods have been used throughout the ages in an attempt to alleviate or cure this pain; these may have been the most appropriate at that time, and were probably seen as "cutting edge". Today they seem at best amusing, and at worst cruel and barbaric.

The earliest concepts in migraine were those of the supernatural, with migraine believed to be due to malevolent beings within the head; treatment based on this idea included incantations and application to the head of substances intended to drive out the demons and spirits (Edmeads, 1991). These were also driven out physically, as in the Neolithic period (8500-7000 BC). The people living in this time used the method of trepanation, a kind of neurosurgery, which involved removing circular chunks of skull so that the spirits causing the headache could escape. Over 50% of the trepanned skulls have shown evidence of healing, indicating a high survival rate for this operation. Although the scientific rationale behind trepanation is not understood, it is surprising that this procedure was performed as a treatment for migraine as late as the mid 17th century (Edmeads, 1991; Rapoport & Edmeads, 2000).

Figure 1.1. Egyptian papyrus (2500 BC), which describes bandaging a clay crocodile (with herbs stuffed into its mouth) to the head of the sufferer and praying

The oldest known medical manuscript, the Ebers Papyrus (dating back to about 1200 BC and discovered in the necropolis of Thebes), contains an ancient Egyptian prescription for migraine based on earlier medical documents including an Egyptian papyrus of 2500 BC. Believing the Gods could cure their ailments, a clay effigy of a sacred crocodile with herbs stuffed into its mouth was firmly bound to the head of the patient by a linen strip (Figure 1.1). Admittedly, this process may have

relieved the headache by collapsing distended cranial blood vessels, which were causing the pain.

Around 400 BC, the ancient Greek physician, Hippocrates, released migraine from the realms of the supernatural by attributing it to vapours rising from the stomach to the head and described, for the first time, the visual symptoms ("aura") of migraine (Edmeads, 1991; Rapoport & Edmeads, 2000). No further progress was reported, but in the 2nd century (AD) Galen wrote of "a painful disorder affecting approximately one-half of the head" (Critchley, 1967). His term for this, "hemicrania", was gradually transmuted into "migraine". Galen, like Hippocrates, believed that this headache was caused by vapours rising from the stomach to the head (Critchley, 1967). The hippocratic/galenic concept of migraine survived into the 17th century, when Thomas Willis published in 1664 his hypothesis that "megrim" was due to dilatation of blood vessels within the head (the first enunciation of a vascular theory) (Edmeads, 1991; Rapoport & Edmeads, 2000). In the years to follow, migraine intensity was decreased by a compression of the superficial temporal artery. In the 19th century, however, the vascular origin of migraine was undermined by a conflicting theory that the prime event was a neurological dysfunction. Thus, in 1873, Edward Liveing proposed that migraine was due to "nerve storms evolved out of the optic thalamus" (Edmeads, 1991). Like the vascular theory, there was nothing but conjecture to support this neurogenic theory (Edmeads, 1991; Rapoport & Edmeads, 2000). Towards the end of the 19th century attempts were made to reconcile both theories. Thus, Moebius stated in 1898 that "parenchyma is the master, circulation the servant", and that both brain and blood vessels dysfunctions were necessary to produce an attack of migraine (Edmeads, 1991). Almost simultaneously, ergot (the product of the fungus Claviceps purpurea that grows upon rye) was introduced in 1884 by W.H. Thomson as an effective remedy for migraine (Thompson, 1894); physicians, however, were aware of the intoxication risk when taken frequently (ergotism or St. Antony's Fire), with descriptions dating back to the Middle Ages (Peroutka, 1995). Ergotism is characterised by gangrene on the feet, legs, hands and arms due to a potent and long-lasting vasoconstriction. Thus, the introduction of ergot and the subsequent isolation of the first pure ergot alkaloid, ergotamine, by Stoll in 1920 (Stoll, 1920), represented a remarkable accomplishment as the beginning of an effective therapy for the treatment of migraine. However, the wide array of

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cardiovascular unwanted effects produced by this ergot (Villalón *et al.*, 2002) prompted the search for more selective antimigraine agents. These attempts ultimately led to the development of sumatriptan as the first selective $5\text{-}HT_1$ receptor agonist effective in the acute treatment of migraine (Feniuk *et al.*, 1991; Humphrey & Feniuk, 1991). However, its short half-life and low oral bioavailability stimulated the development of compounds with longer half-life and higher oral bioavailability, presently known as "second-generation triptans" (Goadsby *et al.*, 2002b).

1.2 Epidemiology

Migraine is a public health problem that has major effects on the individual sufferer, his/her surrounding environment (including family and work) and society. Moreover, the impact of migraine on health care utilisation is well marked and it has been reported that 1% of all visits to physicians (over 10 million visits a year in U.S.A. only) were for headache (Silberstein & Silberstein, 1990). Migraine affects a substantial proportion (16%) of the population (Rasmussen *et al.*, 1991) and is more prevalent in females than in males (15-18% vs. 6%) (Stewart *et al.*, 1992). The incidence of migraine begins earlier in males than in females, and Migraine With Aura begins earlier than Migraine Without Aura (Stewart *et al.*, 1993).

1.3 Co-morbidity

Migraine is co-morbid with a number of neurological and psychiatric disorders, including, amongst others, stroke, epilepsy, depression and anxiety disorders (Low & Merikangas, 2003). Understanding the co-morbidity related with migraine is important in diagnosing and treating this syndrome (Low & Merikangas, 2003). For example, the association between migraine and stroke is well described, as strokes in younger age groups were attributed to migraine (Schwaag *et al.*, 2003); moreover, stroke appears more often with migraine with aura than migraine without aura (Rothrock *et al.*, 1993; Welch, 1994). Analogous to stroke, the median prevalence of epilepsy in migraine patients (6%) exceeds the population prevalence (0.5%) (Andermann, 1987; Hauser *et al.*, 1991). The risk of getting migraine attacks is higher with partial and generalised seizures and highest in post-traumatic epileptics (Lipton *et al.*, 1994; Petzold, 2003). Migraine is also co-morbid with major depression, anxiety and panic disorders (Merikangas *et al.*, 1990; Breslau *et al.*,

1991). The lifetime rates for affective and anxiety disorders are elevated in migraineurs and, in patients with psychiatric disorders, anxiety precedes the onset of a migraine attack, whereas the onset of depression usually follows migraine (Merikangas *et al.*, 1990). Moreover, migraine with aura was more strongly associated with various psychiatric disorders than migraine without aura (Breslau *et al.*, 1991).

1.4   Diagnostic criteria

1.4.1   Based on clinical features

Migraine is a neurovascular disorder, diverse in its expression, complex in manifestation and with an elusive pathophysiology (Villalón *et al.*, 2002). Migraine is characterised by intense, throbbing and pulsatile headache, which is often unilateral in onset; and accompanied by anorexia, nausea, vomiting and photo-and/or phonophobia; in some are preceded by, or associated with, conspicuous sensory, motor and mood disturbances; and are often familial (Elkind & Friedman, 1962; Villalón *et al.*, 2002). Based on clinical features, migraine can be divided into three different phases namely, Premonitory phase (Phase I: occurs hours or days before the headache), Main attack phase (Phase II: an aura phase precedes or occurs with the headache and headache phase) and Post-drome (resolution) phase (Phase III) (Goadsby *et al.*, 2002b).

(I)   PHASE I: PREMONITORY (PRODROME) PHASE

A trigger, usually unknown, can bring about migraine attacks if an individual is susceptible to migraine (Villalón *et al.*, 2002). About 25% of the patients suffering from migraine have reported symptoms like elation, irritability, depression, hunger, thirst or drowsiness during 24 hours preceding headache, indicating a hypothalamic site for their origin (Goadsby *et al.*, 2002b). The premonitory phenomena occur hours to days before the onset of headache and about 60% of the migraineurs experience these premonitory symptoms. These symptoms are seen both in patients with aura or without aura (Goadsby *et al.*, 2002b).

(II)   PHASE II: MAIN ATTACK PHASE

(i)   Phase IIA: aura phase

The migraine aura is a complex of focal neurological symptoms that precedes or accompanies migraine in about 30% of patients (Ziegler & Hassanein, 1990). Most aura symptoms develop over 5-20 min and last about 60 min. This type of attack is also termed as migraine with aura or classical migraine. The aura symptoms may consist of the following characteristics:

- Visual (flashing jagged lights (photopsia) or visual loss),
- Sensory (pins and needle feeling or numbness),
- Motor (weakness or incoordination),
- Language problems (difficulty in finding or using words),
- Brainstem disturbances (vertigo or double vision).

Almost any symptom and sign of brain dysfunction may be a feature of the aura, but the most common aura is a visual, followed by sensory, aphasic and motor symptoms (Russell & Olesen, 1996). However, the majority of migraineurs do not experience the above associated symptoms: this is generally known as migraine without aura or common migraine (Ferrari, 1998).

(ii)   Phase IIB: Headache Phase

The typical migraine headache is unilateral in onset (bilateral in 40% of cases), throbbing in type, pulsatile in nature, moderate to severe in intensity and aggravated by physical activity (Goadsby *et al.*, 2002b). The pain may occur at any time of the day, but most frequently in the early morning, gradual in onset and peaks, then subsides (Goadsby *et al.*, 2002b). It usually lasts between 4 to 72 hours in adults and 2 to 48 hours in children. If the migraine attack persists more than 3 days, the term "status migrainous" is applied.
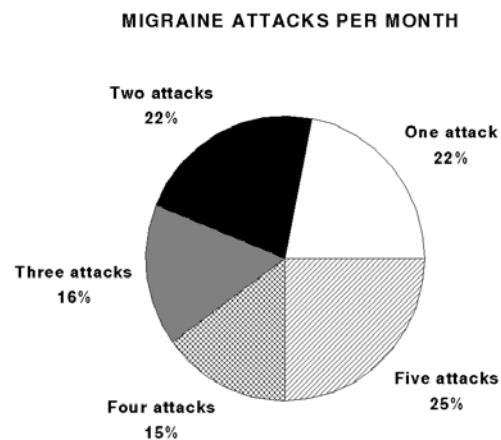
Figure 1.2. Frequency of migraine attacks experienced by migraineurs per month (Silberstein, 1995).

Frequency varies among individuals from a few in a lifetime to several times in a week, with an average of 1-3 a month (Figure 1.2) (Silberstein, 1995; Goadsby *et al.*, 2002b).

PHASE III: POST-DROME (RESOLUTION) PHASE

As the pain lessens, patients feel tired, washed out, irritable and may have impaired concentration, scalp tenderness or mood changes. Some patients feel unusually refreshed or euphoric after the attack while others experience depression and malaise (Goadsby *et al.*, 2002b).

1.4.2   Formal Classification and Diagnostic Criteria for Migraine

The International Headache Society (IHS) formally classified the headaches in order to improve clinical practise and research. In 1988, IHS published the first edition of the International Classification of Headache Disorders (ICHD-I) and it was later redefined in 2004 (ICHD-II) (Olesen *et al.*, 2003b). Migraine was grouped under the primary headaches based on their symptoms, as true aetiological classification is not possible like secondary headaches. This scheme stipulates that certain characteristic features are necessary to establish a diagnosis of migraine.

The IHS system recognises six subtypes of migraine with two major varieties, namely, migraine without aura and migraine with aura. Tables 1.1 and 1.2 show the classification for migraine and diagnostic criteria for migraine with or without aura proposed by the International Headache Society (IHS' 2004) (Olesen *et al.*, 2003b).

Table 1.1. The international classification of migraine (ICHD'2004)

Migraine

1. Migraine without aura

2. Migraine with aura
    2.1 Typical aura with migraine headache
    2.2 Typical aura with non-migraine headache
    2.3 Typical aura without headache
    2.4 Familial hemiplegic migraine (FHM)
    2.5 Sporadic hemiplegic migraine
    2.6 Basilar-type migraine

3. Childhood periodic syndromes that are common precursors of migraine
    3.1 Cyclical vomiting
    3.2 Abdominal migraine
    3.3 Benign paroxysmal vertigo of childhood

4. Retinal migraine

5. Complications of migraine
    5.1 Chronic migraine
    5.2 Status migrainosus
    5.3 Persistent aura without infarction
    5.4 Migrainous infarction
    5.5 Migraine-triggered seizure

6. Probable migraine
    6.1 Probable migraine without aura
    6.2 Probable migraine with aura
    6.3 Probable chronic migraine

Table 1.2. Diagnostic criteria proposed by the International Headache society (ICHD' 2004)

Migraine with aura (Previously used terms: Classic, ophthalmic, hemiparaesthetic, hemiplegic or aphasic, complicated migraine)
A. At least two attacks fulfilling B.
B. At least following three of the following four characteristics

- One or more fully reversible aura symptoms indicating focal cerebral cortical and/or brain stem dysfunction.
- At least one aura symptom develops gradually over more than 4 min, or two or more symptoms occur in succession.
- No aura symptom last more than 60 min.
- Headaches follow aura with a free interval of less than 60 min.

C. At least one of the following

- History, physical and neurological examinations do not suggest secondary headache disorders.
- History and/or physical and/or neurological examinations do suggest such disorder, but it is ruled out by appropriate investigations.
- Such disorder is present, but migraine attacks do not occur for the first time in close temporal relation to the disorder.

Migraine without aura (Previously used terms: Common migraine, hemicrania simplex)
A. At least five attacks fulfilling B-D.
B. Headache attacks lasting 4 to 72 hours (untreated or unsuccessfully treated).
C. Headache has at least two of the following characteristics:

- Unilateral location.
- Pulsatile quality.
- Moderate or severe intensity (inhibits or prohibits daily activities).
- Aggravation by or causing avoidance of routine physical activity (e.g., walking stairs or similar routine physical activity).

D. During headache at least one of the following

- Nausea and/or vomiting.
- Photophobia and phonophobia.

E. At least one of the following

- History, physical and neurological examinations do not suggest secondary headache disorders.
- History and/or physical and/or neurological examinations do suggest such disorder, but it is ruled out by appropriate investigations.
- Such disorder is present, but migraine attacks do not occur for the first time in close temporal relation to the disorder.

1.5   Pathophysiology of Migraine

A migraine attack is believed to be an inherited instability in the brain sensory control system (i.e., hyperexcitable brain); when this system malfunctions either due to accumulation of unknown triggers or other mechanisms, results in migraine headache (Bigal *et al.*, 2002). Based on its clinical features, three distinct phases of migraine can be discerned, namely, a trigger, an aura and a headache phase.

1.5.1   Trigger phase including premonitory symptoms

Although limited information regarding the trigger is available, there is a better conception about the pathophysiology of migraine (Ferrari, 1998; Villalón *et al.*, 2002). Moreover, it is believed that an initiating trigger arises from the brain stem known as "migraine generator" and may also be due to a genetic predisposition (Ophoff *et al.*, 1996; Ferrari, 1998). The subsequent events following the trigger phase leading to the symptoms observed during the aura and headache phases can be explained on the basis of the neurovascular hypothesis (Ferrari & Saxena, 1993b; Villalón *et al.*, 2002).

1.5.2   Aura Phase

As mentioned in the Figure 1.3 (Tfelt-Hansen *et al.*, 2000), once the brain stem gets activated (i.e., the brain generator has been switched on), there is a decrease in the regional cerebral blood flow, possibly following a wave of cortical spreading depression (Goadsby *et al.*, 2002b). When the cerebral blood flow decreases beyond a critical level, the corresponding aura symptoms occur. Most clinicians believe that the migraine aura is due to a neuronal dysfunction rather than ischaemia and it is probably the clinical manifestation of a cortical spreading depression (Olesen, 1991a). The majority of migraine patients do not experience aura, but the following disturbances: (i) scintillating scotoma, flashing of lights that move across the visual field, etc.; (ii) paraesthesias; or (iii) other neurological signs (Goadsby *et al.*, 2002b). The decrease in the cerebral blood flow begins usually in the occipital lobe, but this reduction enlarges and may involve the whole hemisphere. This spreading oligemia does not respect the vascular territories and it is unlikely due to vasoconstriction (Olesen, 1991a).
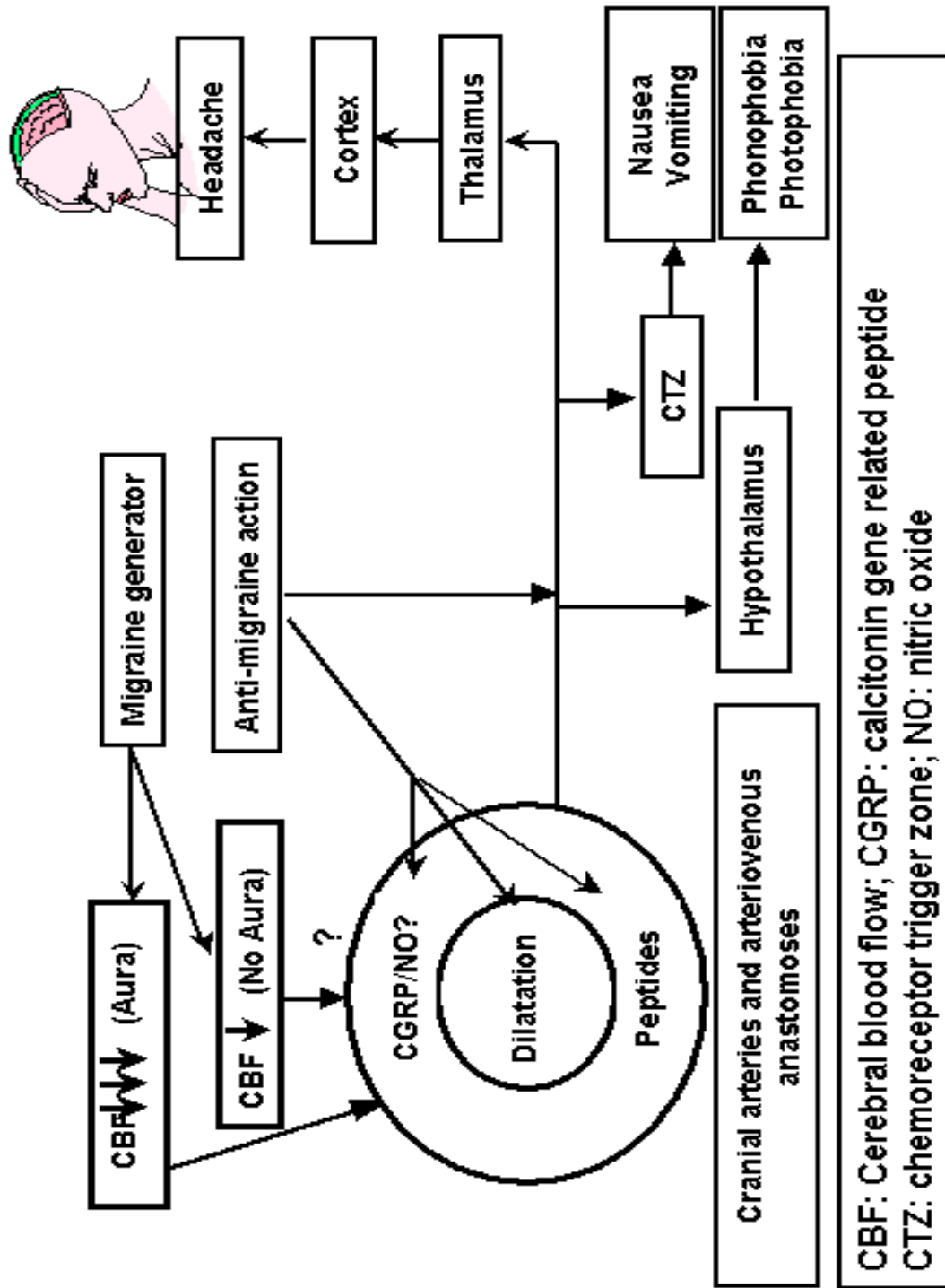
Figure 1.3. Pathophysiology of migraine.

CBF: Cerebral blood flow; CGRP: calcitonin gene related peptide; CTZ: chemoreceptor trigger zone; NO: nitric oxide

1.5.3   Headache Phase

The cerebral oligemia is subsequently followed by a reflex vasodilatation of the cranial blood vessels and arteriovenous anastomoses, probably due to changes in the neuronal activity that innervates the cranial extracerebral blood vessels and arteriovenous anastomoses (e.g., dura mater, base of the skull and scalp region). Tracing studies have shown that the fibres innervating the cerebral blood vessels arise from within the trigeminal ganglion containing several vasoactive neurotransmitters including substance P, calcitonin-gene related peptide (CGRP), 5-hydroxytryptamine (5-HT), vasoactive intestinal peptide (VIP), nitric oxide (NO) and neurokinin A (Goadsby *et al.*, 2002b). This profuse cranial vasodilatation leads to an enhanced blood volume following each cardiac stroke and rapid diastolic run off, with a consequent augmentation in carotid pulsations within the affected blood vessels. These augmented pulsations can then be sensed by so-called "stretch" receptors in the vessel wall thereby activating the perivascular (trigeminal) sensory nerves (De Vries *et al.*, 1999a; De Vries *et al.*, 1999b). This nociceptive information is conveyed to central neurons in the trigeminal sensory nucleus that in turn relays the pain signals to higher centers where headache pain is perceived (Williamson & Hargreaves, 2001; Edvinsson, 2003). In addition, stimulation of trigeminal nerves may also release neuropeptides, thus reinforcing vasodilatation and perivascular nerve activity (Villalón *et al.*, 2002).

Acutely-acting antimigraine compounds constrict dilated cranial extracerebral blood vessels (Saxena & Ferrari, 1989; Feniuk *et al.*, 1991; Ferrari & Saxena, 1993a) and inhibit neuropeptide release, plasma protein extravasation across dural blood vessels (Buzzi *et al.*, 1992) and impulse transmission within the trigeminovascular system (Goadsby *et al.*, 2002b).

1.6   Experimental models for acutely-acting antimigraine drugs

The experimental models currently known for the discovery and development of antimigraine drugs are based on the vascular or neurogenic involvement in migraine (De Vries *et al.*, 1999a): (i) vasoconstriction of the dilated extracranial blood vessels including carotid arteriovenous anastomoses (e.g., carotid vasculature or isolated blood vessels; vascular hypothesis); (ii) inhibition of the trigeminal system (e.g., blockade of plasma protein extravasation and/or central trigeminal inhibition; neurogenic hypothesis); and (iii) combination of both (e.g., inhibition of neurogenic vasodilatation).

1.6.1   Experimental models based on the vascular involvement

(i)   Constriction   of   carotid   arteriovenous   anastomoses (Figure 1.4)   in
           anaesthetised animals

Although a complete understanding of the migraine pathogenesis remains elusive, there seems to be little doubt that the dilatation of cranial blood vessels, including carotid arteriovenous anastomoses, is involved in the headache phase of migraine (De Vries *et al.*, 1999a).   In addition to headache, migraine patients also experience facial paleness, reduction in the facial temperature, increase in the temporal artery pulsations and swelling of the frontal vein on the side of the headache (Drummond & Lance, 1983; Drummond & Lance, 1984). Based on these findings, Heyck (Heyck, 1969) investigated the potential underlying mechanisms involve in migraine, by measuring the oxygen saturation difference between the arterial
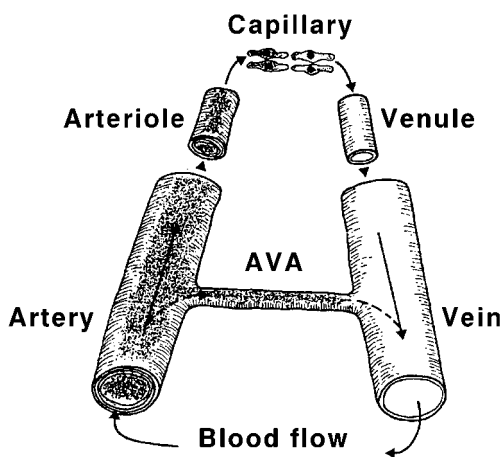
Figure 1.4. A schematic representation of an arteriovenous anastomosis

(femoral) and venous (external jugular) blood samples (A-V SO_2 difference) during and after the headache phase of migraine and compared it with the healthy control groups (Heyck, 1969). Interestingly, he observed that the A-V SO_2 difference was

abnormally decreased during the headache phase of migraine, likely due to dilatation of the carotid arteriovenous anastomoses, and this decrease was normalised after spontaneous or drug-induced (ergotamine) alleviation of the headache (Heyck, 1969).

Arteriovenous anastomoses are precapillary communications between the arteries and veins (Figure 1.4); they are predominantly located in the head skin, ears, nasal mucosa, eyes and dura mater in several species, including humans and pigs (Saxena, 1995). In conscious pigs, the arteriovenous anastomoses are constricted being under a strong influence of the sympathetic neuronal tone, thereby shunting only a small (<3%) fraction of the total carotid blood flow (Hales, 1974). In contrast, under pentobarbital anaesthesia, ~80% of the total carotid blood flow is shunted via arteriovenous anastomoses into the jugular venous circulation (Den Boer *et al.*, 1993). Consequently, opening of the carotid arteriovenous anastomoses during migraine shunts a large quantity of oxygenated blood directly into the veins thereby resulting in facial pallor, lowering of skin temperature and increase in vascular pulsations (Saxena, 1995). This increase in vascular pulsations stimulate the so-called 'stretch receptors' present in the wall of blood vessels, with ensuing activation of perivascular trigeminal nerves containing peptides (e.g. CGRP) (De Vries *et al.*, 1999a; De Vries *et al.*, 1999b). The fifth cranial nerve conveys nociceptive information to central trigeminal nuclei that in turn relay the pain signals to higher centres where headache pain is perceived (Williamson & Hargreaves, 2001; Edvinsson, 2003).

In line with the above findings, it is reasonable to assume that the constriction of dilated carotid arteriovenous anastomoses may abort migraine. Therefore, we developed an animal experimental model using radioactive microspheres to determine carotid arteriovenous anastomostic blood flow and the effects of antimigraine drugs on carotid arteriovenous anastomoses (Saxena, 1990; Saxena, 1995). Over the years, this model has proven predictive of antimigraine activity in the clinic (Saxena, 1995). Another major advantage of this model is that one can simultaneously study different vascular beds in order to evaluate the cranioselectivity of current or prospective antimigraine drugs (De Vries *et al.*, 1999a; De Vries *et al.*, 1999b). Based on this notion, we have previously shown that conventional antimigraine agents like ergotamine and sumatriptan as well as second-generation triptans potently constrict the porcine carotid arteriovenous anastomoses (Willems *et al.*, 1998; De Vries *et al.*, 1999a; Tom *et al.*, 2002). Moreover, we have

recently demonstrated that α_1- and α_2-adrenoreceptors mediate porcine carotid vasoconstriction and suggested that selective agonists at these receptors might provide a promising novel avenue for the development of acute antimigraine drugs (Willems *et al.*, 2003).

Several lines of evidence indicate that CGRP, a potent vasodilator released from the trigeminal sensory nerves may play an important role in the pathophysiology of migraine (Goadsby *et al.*, 2002b). Indeed, CGRP receptors are widely distributed in several vascular beds including the carotid vasculature (Gardiner *et al.*, 1990; Van Gelderen *et al.*, 1995; Shen *et al.*, 2001). Moreover, triptans abort migraine not only by constricting the dilated cranial blood vessels via 5-HT$_{1B}$ receptors, but also by inhibiting CGRP release by activation of 5-HT$_{1D}$ receptors (Goadsby *et al.*, 2002b). Therefore, it is admissible to propose that CGRP receptors may be involved in the vascular tone of the carotid circulation, which may provide a novel target for developing new antimigraine compounds (Edvinsson, 2003). The recently introduced potent and selective CGRP receptor antagonist, BIBN4096BS (Doods *et al.*, 2000), may be a useful as a pharmacological tool to evaluate the potential role of CGRP receptors in migraine (Edvinsson, 2003). The following chapters (Chapters 2 and 3) will discuss the effects of BIBN4096BS on porcine carotid haemodynamics as well as its cardiac output distribution.

(ii)   Contraction of isolated cranial blood vessels

Several *in vitro* studies using a number of isolated blood vessels have shown that the acute antimigraine compounds contract these blood vessels via 5-HT$_1$ receptors, (De Vries *et al.*, 1999a; Villalón *et al.*, 2002). This contractile effect is more marked in cranial blood vessels than in peripheral blood vessels where 5-HT$_2$ receptors are predominant (Longmore *et al.*, 1997). It is noteworthy that the pharmacological profile of the above contractile 5-HT$_1$ receptors correlates with the 5-HT$_{1B}$, but not the 5-HT$_{1D}$ or 5-ht$_{1F}$ receptor subtypes (De Vries *et al.*, 1998; Verheggen *et al.*, 1998; Cohen *et al.*, 1999). In addition, dipeptide CGRP receptor antagonists, such as BIBN4096BS and compound 1, potently antagonise CGRP-induced vasorelaxations in cerebral arteries (Edvinsson, 2001a; Edvinsson, 2002; Moreno *et al.*, 2002; Verheggen *et al.*, 2002).

1.6.2   Experimental models based on the neurogenic involvement

The basic perception behind the development of neurogenic models for migraine is that migraine pain is due to a sterile neurogenic inflammation within the meninges and consequent activation of trigeminal nerve terminals (Williamson & Hargreaves, 2001).   The activated trigeminal nerves release several neuropeptides (including substance P, neurokinin A and CGRP), which cause subsequent features of migraine rather than merely dilatation of cranial blood vessels (Goadsby *et al.*, 2002b). Therefore, the efficacy of an antimigraine drug is believed to be due to a presynaptic action on sensory nerves thereby inhibiting the neuropeptide release and the process and/or consequences of "neurogenic inflammation" (Buzzi *et al.*, 1991; Buzzi *et al.*, 1992).   Moreover, mechanisms, which do not seem to be mediated solely by the 5-HT_{1B} receptor have also been implicated in migraine relief (Goadsby, 1998).   These mechanisms include inhibition of the trigemino-vascular system peripherally and/or centrally (Goadsby, 1998; May *et al.*, 1998).

(i)   Inhibition of plasma protein extravasation after trigeminal stimulation

Migraine pain is believed to be a form of sterile neurogenic inflammation, which is characterised by plasma proteins extravasation across the dura mater and associated structural changes in the dura mater, such as increases in endothelial permeability and mast cell degranualation (Williamson & Hargreaves, 2001).   The concept of plasma protein extravasation gained importance in migraine pathogenesis following a study demonstrating plasma protein extravasation following antidromic stimulation of trigeminal ganglion/sensory nerve in rats and guinea pigs (Moskowitz, 1993). Clinically effective antimigraine agents, such as ergots, triptans, opioids and valporate, inhibited this sterile neurogenic inflammation, suggesting that plasma protein extravasation inhibition could be predictive of antimigraine therapeutic activity (Moskowitz, 1993).

Based on this finding, the compounds inhibiting plasma protein extravasation were investigated as new approaches in migraine treatment.   The conventional antimigraine drug, sumatriptan, inhibits plasma protein extravasation and this effect was attenuated by the 5-$HT_{1B/1D}$ receptor antagonist GR127935 in both rats and guinea pigs, implying the involvement of 5-$HT_{1B/1D}$ receptor subtypes (Williamson & Hargreaves, 2001).   However, in mice this effect resembles 5-HT_{1B} receptors whereas

in guinea pigs and rats, it is a 5-HT$_{1D}$-mediated effect (Shepheard *et al.*, 1997; Yu *et al.*, 1997; Williamson & Hargreaves, 2001). Although triptans have high affinity for both 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor subtypes, they may also act on other subtypes (Williamson & Hargreaves, 2001). In this respect, plasma protein extravasation inhibition can also be induced by 5-carboxamidotryptamine and CP122288 in 5-HT$_{1B}$ receptor knockout mice and this effect is not prevented by GR127935 in guinea pigs (Yu *et al.*, 1996; Yu *et al.*, 1997). Moreover, CP122288 shows a higher potency than sumatriptan in rats and this did not correlate with its affinity for 5-HT$_{1B}$ or 5-HT$_{1D}$ receptors, suggesting that at least part of the CP122288 action may be via 5-ht$_{1F}$ receptors, where it displays a high affinity (Shepheard *et al.*, 1997; Williamson & Hargreaves, 2001). Moreover, a number of 5-HT$_1$ receptor agonists that inhibit plasma protein extravasation in guinea pig dura mater displaying a rank order of potency that correlates with their affinity towards 5-ht$_{1F}$ rather than 5-HT$_{1B}$ or 5-HT$_{1D}$ receptor subtypes (Williamson & Hargreaves, 2001). Based on this finding, a selective 5-ht$_{1F}$ receptor agonist, LY344864, was developed (Johnson *et al.*, 1997). This compound, besides proving effective in inhibiting plasma protein extravasation produced by trigeminal ganglion stimulation in rats, was apparently effective in acute migraine treatment (Goldstein *et al.*, 2001b). Despite the clinical effectiveness of LY344864, it was not clear whether plasma protein extravasation inhibition was responsible for its antimigraine properties. Moreover, LY344864 inhibits the activation of brainstem neurons in response to the stimulation of dura mater as well as the c-fos expression in trigeminal nucleus caudalis; this suggests that the primary mechanism of LY344864 is central (i.e. interruption of the ascending pain pathways) rather than peripheral (inhibition of plasma protein extravasation) (Williamson & Hargreaves, 2001). Similarly, the selective 5-HT$_{1D}$ receptor agonist PNU-142633F, which blocks plasma protein extravasation in guinea pigs (Cutrer *et al.*, 1999), was ineffective in migraine treatment (Cutrer *et al.*, 2000). Furthermore, other studies have shown that plasma protein extravasation can be inhibited by the CGRP receptor antagonist CGRP $_{(8-37)}$ (O'Shaughnessy & Connor, 1994; Brandli *et al.*, 1996).

Importantly, plasma protein extravasation models do not always predict antimigraine efficacy (Goadsby, 2000) as clearly evidenced by the failure of several compounds in clinical trials, including: (i) the NK$_1$ receptor antagonist, lanipetant (Goldstein *et al.*, 1997); (ii) specific plasma protein extravasation inhibitors such as

CP122,288 and 4991W93 (Roon *et al.*, 2000); (iii) the $ET_{A/B}$ receptor antagonist bosentan (May *et al.*, 1996); and (iv) the neurosteroid ganaxolone (Data *et al.*, 1998). Moreover, the clinical antimigraine predictability of plasma protein extravasation assays became questionable following an elegant clinical study in migraine showing no increases in retinal or choroid permeability (May *et al.*, 1998); this contrasts with the increase in retinal or choroid permeability following trigeminal ganglion stimulation in rats (Williamson & Hargreaves, 2001).

(ii)   Inhibition of cranial vasodilatation (carotid, dural and cortical) induced by trigeminal stimulation

Electrical stimulation of the trigeminal nerve in humans evokes the release of CGRP in cranial venous blood (Goadsby *et al.*, 2002b). Moreover, during the headache phase of migraine, plasma levels of CGRP, but not substance P, were elevated in the jugular venous blood (Goadsby *et al.*, 2002b). Therefore, CGRP released from trigeminal sensory nerves that innervate cranial blood vessels produces vasodilatation thereby causing headache (Williamson & Hargreaves, 2001). Based on this, several animal models were developed to demonstrate cranial vasodilatation associated to CGRP release produced by trigeminal stimulation as well as to study the effects of antimigraine drugs on it (Williamson & Hargreaves, 2001). It is known that triptans attenuate cranial vasodilatation induced by trigeminal stimulation as well as CGRP release in rats (Williamson & Hargreaves, 2001). However, carotid vasodilatation in guinea pigs following trigeminal ganglion stimulation is mediated by vasoactive intestinal peptide, which was not amenable to blockade by antagonists at CGRP or tachykinin receptors (Beattie & Connor, 1994; Raval *et al.*, 1999).

Therefore, another model was developed in which trigeminal sensory Aδ-fibres were stimulated following short, low intensity electrical stimulation (which releases CGRP only); the dural blood vessel diameter was measured by an intravital microscope through a closed cranial window (Williamson & Hargreaves, 2001; Akerman *et al.*, 2003). Electrical stimulation of this cranial window as well as intravenous infusion of substance P and α-CGRP in rats evoked an increase (80%) in dural blood vessel diameter (Shepheard *et al.*, 1997; Williamson & Hargreaves, 2001). Interestingly, the NK_1 receptor antagonist, RP 67580 clearly antagonised substance P-induced vasodilatation, but not the neurogenic vasodilatation (Williamson

& Hargreaves, 2001). However, the CGRP receptor antagonist $CGRP_{(8-37)}$ completely antagonised the vasodilatation induced by both α-CGRP and neurogenic stimulation (Akerman *et al.*, 2003); this suggests that the neurogenic vasodilatation is mediated by endogenous CGRP released from trigeminal sensory nerves. This observation is consistent with clinical data showing that CGRP, but not substance P, levels are elevated during the headache phase of migraine (Goadsby *et al.*, 1990).

Significantly, triptans attenuated the neurogenic dural vasodilatation following trigeminal stimulation, probably via presynaptic inhibition of CGRP release (Williamson & Hargreaves, 2001). This observation mimicked clinical situations since sumatriptan normalised elevated plasma CGRP levels with resolution of the headache (Williamson & Hargreaves, 2001). It was suggested that the above inhibitory effect of sumatriptan is mediated via prejunctional $5-HT_{1B}$ receptors in rats and $5-HT_{1D}$ receptors in guinea pigs, cats and humans (Williamson & Hargreaves, 2001).

(iii)   Central trigeminal neuronal inhibition

The importance of brainstem in the pathogenesis of migraine was emphasised following its activation during migraine attacks (Bahra *et al.*, 2001). During migraine, blood flow was increased in the cerebral hemispheres (cingulate, auditory and visual cortex) as well as in brain stem (Bahra *et al.*, 2001). Sumatriptan relieved the headache and other symptoms as well as reversed the increase in cerebral blood flow, but not in brain stem; this indicates that persistent brain stem activation is due to other factors including increased activity of the endogenous antinociceptive system (Bahra *et al.*, 2001). Moreover, the brain stem activation may be inherent of the migraine process itself, and continuous activation of brain stem (despite symptom resolution by sumatriptan) may account for the headache recurrence (Bahra *et al.*, 2001).

Based on this finding, animal migraine models were developed to study c-fos activation of the trigeminal nucleus caudalis; interestingly, this effect was not altered by sumatriptan (Goadsby, 1997a; Goadsby, 1997b; Goadsby & Hoskin, 1997; Goadsby & Knight, 1997). However, the second generation triptans, such as zolmitriptan (Goadsby & Boes, 2001), naratriptan (Donaldson *et al.*, 2002) and eletriptan (Donaldson *et al.*, 2002; Lambert *et al.*, 2002) inhibited the action potentials generated in the trigeminal nucleus caudalis after superior saggital sinus stimulation in

cats and dural stimulation in rats (Cumberbatch *et al.*, 1997). This difference in effects could be due to the poor central penetrating effects of sumatriptan (Ferrari & Saxena, 1993a; Ferrari & Saxena, 1993b; Saxena & Tfelt-Hansen, 1993) as compared to second generation triptans, which are known to have central inhibitory effects (Saxena & Tfelt-Hansen, 2000; Goadsby *et al.*, 2002b). Consequently, it has been argued that the blood brain barrier may be disrupted during migraine (Harper *et al.*, 1977); indeed, under experimental disruption of the blood brain barrier by hyperosmolar mannitol, sumatriptan produced central inhibitory effects (Shepheard *et al.*, 1995). However, there is little or no evidence for a disrupted blood brain barrier based on computerised tomography or MRI findings in migraine patients (Alvarez-Cermeno *et al.*, 1986; Hamalainen *et al.*, 1996).

Several lines of pharmacological evidence indicate that potent antimigraine agents act on the second order trigeminal neurons to reduce cell activity, suggesting that trigeminocervical complex neurons in the caudal brain stem could be a possible target for antimigraine activity (Bahra *et al.*, 2001). It is likely that this central inhibitory effect is mediated by $5\text{-HT}_{1B/1D}$ receptors since the central inhibitory effect of eletriptan in cats is amenable to blockade by GR127935. In addition, the involvement of 5-HT_{1D} receptors rather than 5-HT_{1B} receptors is crucial for this effect (Donaldson *et al.*, 2002; Lambert *et al.*, 2002). Moreover, CGRP mediates sensory nerve transmission between the first and second order afferent input from the cranial blood vessels, and CGRP receptor antagonists may attenuate sensory nerve transmission (Gulbenkian *et al.*, 2001; Williamson & Hargreaves, 2001; Conner *et al.*, 2002; Goadsby *et al.*, 2002b; Poyner *et al.*, 2002; Smith *et al.*, 2002). Recently, adenosine A_1 receptors were localised in human trigeminal ganglia, suggesting a potential usage of adenosine A_1 receptor agonists to inhibit trigeminal nociception (Welch, 2003).

1.7   Management of migraine

The drugs used in the treatment of migraine can be divided into two groups: agents that abolish the acute migraine headache (acute antimigraine drugs; e.g. ergotamine and sumatriptan) and agents aimed at its prevention (prophylactic drugs; e.g. methysergide). Patients who experience frequent headaches may require both forms of treatment.

1.7.1 Acute treatment

Though several acutely acting antimigraine drugs are available, administration of these drugs depends upon: (i) severity; (ii) frequency of the attacks; (iii) associated symptoms; and (iv) co-morbid conditions interrelated with a migraine attack (Villalón *et al.*, 2002). Acutely acting antimigraine drugs attempt to abort the headache and they can be specific or non-specific in action. Non-specific medications control the pain and associated symptoms of migraine or other pain disorders; in contrast, the specific medications control the headache attack, but are not useful for non-headache pain disorders (Goadsby *et al.*, 2002b).

(I)   NON-SPECIFIC MEDICATIONS

Non-specific medications are prescribed for mild to moderate headaches; the most commonly used drugs include non-steroidal antiinflammatory drugs (NSAIDs; e.g. aspirin or acetaminophen) either alone or combination with caffeine. NSAIDs are the most popular agents because, in addition to being cheap, effective and easy to administer, they allow a control over own therapy. Unfortunately, they produce headache after a long-term use (Villalón *et al.*, 2002). For associated symptoms (e.g. nausea or vomiting), antiemetics such as domperidone or metoclopromide are administered (Goadsby *et al.*, 2002b).

(II)   SPECIFIC-MEDICATIONS

Specific antimigraine drugs abort the migraine headache by constricting the dilated extracranial blood vessels, including the external carotid bed (Olesen, 1991a; Olesen, 1991b; Rasmussen *et al.*, 1991; Saxena & Den Boer, 1991). The most commonly used specific antimigraine compounds are ergot alkaloids and triptans.

a.   Ergot alkaloids

Ergotamine and its derivative dihydroergotamine are used to treat moderate to severe migraine attacks, particularly if NSAIDs fail to alleviate the headache (Saxena & Den Boer, 1991). Dihydroergotamine has fewer side effects than ergotamine and they are effective in most of the patients with low recurrence rate. Ergot and its derivatives are contraindicated in patients with uncontrolled hypertension, hepatic or renal failure, vascular disease (coronary, cerebral and/or peripheral) and in pregnancy (Villalón *et al.*, 2002).

b.   Selective 5-HT agonists

Based on the vascular involvement in migraine and with the supporting role of 5-HT in the migraine pathogenesis (Saxena & Tfelt-Hansen, 2000), a new tryptamine derivative was synthesised to achieve selectivity at the craniovascular 5-HT_1 like receptors; this search culminated in the design and development of sumatriptan, a selective $5\text{-HT}_{1B/1D}$ receptor agonist (Humphrey *et al.*, 1989). Sumatriptan constricts cranial arteries and displays much less activity in other vascular systems (Dahlöf & Saxena, 2000; MaassenVanDenBrink *et al.*, 2000a; MaassenVanDenBrink *et al.*, 2000b; Saetrum Opgaard *et al.*, 2000; Saxena & Tfelt-Hansen, 2000; Tfelt-Hansen *et al.*, 2000; Villalón *et al.*, 2002). Sumatriptan relieves headache, nausea, vomiting and restores the persons back to normal situations (Goadsby *et al.*, 2002b). Sumatriptan is available in subcutaneous or nasal spray forms; they are used for immediate relief of headache, while the oral form is used in patients with gradual onset of headache (Goadsby *et al.*, 2002b). Despite its advantage over other conventional antimigraine compounds, it has low oral bioavailability, short half-life and high headache recurrence (up to 40%) and is contraindicated in patients suffering from coronary arterial disease (Maassen VanDenBrink *et al.*, 1999; Goadsby *et al.*, 2002b). Therefore, in order to overcome these limitations, several new $5\text{-HT}_{1B/1D}$ receptor agonists (referred to as second-generation triptans) have been developed with an action similar to that of sumatriptan, but different in their pharmacokinetic properties (particularly higher oral bioavailability, longer half-life, low headache recurrence, etc.).

1.7.2   Preventive (prophylactic) treatment

Patients who experience migraine attacks that are frequent, severe, long lasting, unresponsive to acute antimigraine agents and that cause substantial disability are the candidates for preventive therapy (Villalón *et al.*, 2002). Preventive antimigraine drugs are taken every day (whether or not the headache is present), to reduce the frequency and severity of migraine attacks (Villalón *et al.*, 2002). The mechanism by which the preventive antimigraine drugs work is still unclear, but they are believed to modify the sensitivity of the brain that underlies migraine pathogenesis (Goadsby *et al.*, 2002b). The commonly used preventive antimigraine agents include, β-adrenergic antagonists, calcium channel blockers, antidepressants, serotonin antagonists,

anticonvulsants and NSAIDs (Goadsby *et al.*, 2002b). Moreover, botulinum toxin (type A) appears to be safe, tolerable and possibly effective drug for migraine prevention, with almost no systemic adverse events (Ashkenazi & Silberstein, 2003). Interestingly, angiotensin converting enzyme receptor blocker candesartan appears to be effective and highly tolerable in the prevention of migraine, but needs to be further evaluated (Ashkenazi & Silberstein, 2003). If preventive medication is indicated, the drug should be chosen from one of the above categories based on side-effect profiles and co-existent morbidities (Silberstein & Lipton, 1994; Silberstein, 1995). It has been reported that on average, about two-thirds of the patients with the above preventive medications had a significant reduction (50%) in the frequency and severity of the attacks (Goadsby *et al.*, 2002b). Natural products such as riboflavin (Vitamin B$_2$: used for mitochondrial dysfunction), niacin, magnesium and leukotriene antagonists (montelukast) have shown some promising results in reducing the frequency and severity of migraine attacks (Bigal *et al.*, 2002; Velling *et al.*, 2003).

In conclusion, though the pathophysiological basis of migraine still remains elusive, the development of migraine models has remarkably contributed to the understanding of migraine pathophysiology and to the development of more effective antimigraine drugs. Hopefully, the advent of novel antimigraine compounds and the introduction of molecular biology techniques will shed further light on this complex scenario.

1.8 Calcitonin gene-related peptide

1.8.1 Introduction

The calcitonin family of peptides comprises five members, namely calcitonin, amylin, calcitonin gene-related peptide (CGRP; two forms α-CGRP and β-CGRP), and adrenomedullin (Poyner & Marshall, 2001). They all have a six-aminoacid ring structure (seven for calcitonin) close to their N-terminal, formed by an intermolecular disulfide bond. This is followed by a region of potential amphipathic α-helix and C amidated terminals (Poyner & Marshall, 2001). These peptides are widely distributed and induce multiple biological effects, such as potent vasodilatation (CGRP and amylin), reduction in nutrient intake (amylin) and decrease in bone resorption (calcitonin) (Poyner & Marshall, 2001). Due to their similarities in structure and biological activities, it is suggested that these peptides interact with similar G-protein coupled receptors (Poyner & Marshall, 2001).

CGRP was first identified in 1983 in rats, where serially transplanted rat cells from a medullary thyroid carcinoma showed a spontaneous ability to switch from a high to a low calcitonin (calcitonin) producing state, by increasing the size of the *mRNA* (Rosenfeld *et al.*, 1983); this was later determined to be an "altered *mRNA*" derived from the calcitonin/CGRP gene (Wimalawansa, 2001). The alternative splicing of this primary *mRNA* transcript of the calcitonin/CGRP gene, which is encoded on the short arm of chromosome 11p14, will lead to the translation of CGRP and calcitonin gene in a tissue specific manner (Wimalawansa, 2001). For example, in the central nervous system (CNS), splicing of the calcitonin/CGRP gene *mRNA* transcript will produce CGRP, whereas in the C cells of the thyroid gland, calcitonin is predominantly formed (Hoppener *et al.*, 1985).

In 1984, based on the sequence of rat α-CGRP, a similar peptide (human CGRP-α; hα-CGRP) from a human medullary carcinoma was demonstrated (Morris *et al.*, 1984). Subsequently, a second CGRP gene (β-CGRP), also located in chromosome 11 and thought to have arisen by the exon duplication, was identified by further analysis of the rat and human cDNA clones (Amara *et al.*, 1985; Wimalawansa, 1996). The r-α-CGRP differs from r-β-CGRP by one aminoacid and the h-β-CGRP differs by three aminoacids from homologous h-α-CGRP (Steenbergh

et al., 1984; Amara *et al.*, 1985); both α-form and β-form of CGRP are very similar in their biological activities (Poyner & Marshall, 2001).

1.8.2   Molecular genetics

A schematic representation of the human α-calcitonin/CGRP gene is illustrated in Figure 1.5. The α-calcitonin/CGRP gene (located in chromosome 11) contains six exons of which first three exons are constitutively spliced in both *mRNA*s (calcitonin and CGRP). The exon I is untranslated, whereas the exons II and III code for the signal peptide and N-terminal propeptide, respectively. The calcitonin and CGRP sequences are localised in exons IV and V, respectively; exon VI is a part of α-CGRP *mRNA*, but untranslated (Wimalawansa, 1996). The primary *mRNA* transcript includes all six exons, and the calcitonin or CGRP *mRNA* is formed subsequently (see Figure 1.1) (Steenbergh *et al.*, 1984). The splicing of the first three exons with exons V and VI produces CGRP containing *mRNA*. The exon V encodes CGRP, while the exon VI encodes the 3'-untranslated region of the CGRP *mRNA* and polyadenylation (poly A) signal (Wimalawansa, 1996). This *mRNA* is translated to generate the pro-CGRP peptide, which is subsequently cleaved at the paired dibasic aminoacids to release the 37-aminoacid CGRP (Amara *et al.*, 1985).

The organization of the β-CGRP gene in the chromosome 11 is similar to that of α-calcitonin/CGRP gene (Steenbergh *et al.*, 1984). While exon I is untranslated, exon II encodes for the signal peptide and exon III, which is 92% homologous to exon II of the α-calcitonin/CGRP gene, encodes for the N-terminal propeptide. The exon IV of the β-CGRP gene is 67% homologous to the same region of the α-calcitonin/CGRP gene that gives rise to calcitonin. The exon IV lacks polyadenylation, thereby preventing the alternative splicing. Consequently, the transcript from this gene produces only CGRP; therefore it is considered as a pseudogene for calcitonin (Proudfoot & Brownlee, 1976; Alevizaki *et al.*, 1986).
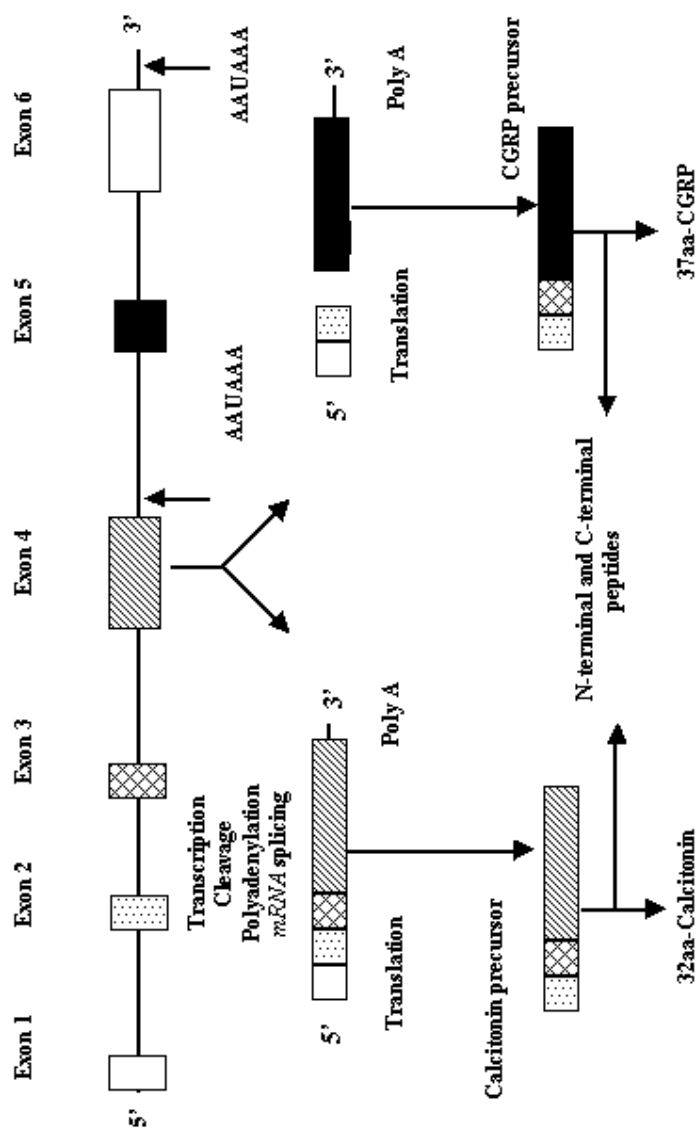
Figure 1.5. Alternative splicing of the calcitonin gene generating calcitonin and CGRP in a tissue specific manner

1.8.3   Structure

All species variants of CGRP have 37 aminoacids, constituted as a single polypeptide chain (Bell & McDermott, 1996). The structure of α-CGRP (Figure 1.6) comprises a N-terminal disulfide bridge between the positions 2 and 7 (Cys2 and Cys7), a well-defined α-helix between the residues 8 and 18. This is followed by either a β- or a γ-turn in the region of residues 19 to 21 and phenylalanylamide C-terminus in the regions of residues 28 and 30, and also in 32 and 34 (Conner *et al.*, 2002). The β-CGRP differs from the α-form by one and three aminoacids in rats and humans, respectively. CGRP shares ~50% homology in their sequence of aminoacids with amylin and some homology with amylin (Chantry *et al.*, 1991; Kitamura *et al.*, 1993).

1.8.4   Structure-activity relationships

It has been suggested that an intact peptide is required for the full biological activity of a CGRP molecule (Zaidi *et al.*, 1990). The N-terminal loop (disulphide-bonded loop) is principally involved in triggering the signal transduction and receptor activation (Conner *et al.*, 2002). The α-helix plays an important role in the interaction of the molecule with the receptor (receptor binding) and its deletion causes approximately 100 fold loss of affinity, (Conner *et al.*, 2002). The residues of 19-27 are necessary as a spacer or hinge region. The C-terminal region (residues 28-37) shows a weak binding to CGRP receptors. However, by making a few more aminoacid substitutions, a high affinity antagonist such as $CGRP_{(8-37)}$ can be generated (Conner *et al.*, 2002). The C-terminal region is probably necessary for the peptide to assume the right conformation in the interaction with its receptor (Conner *et al.*, 2002).
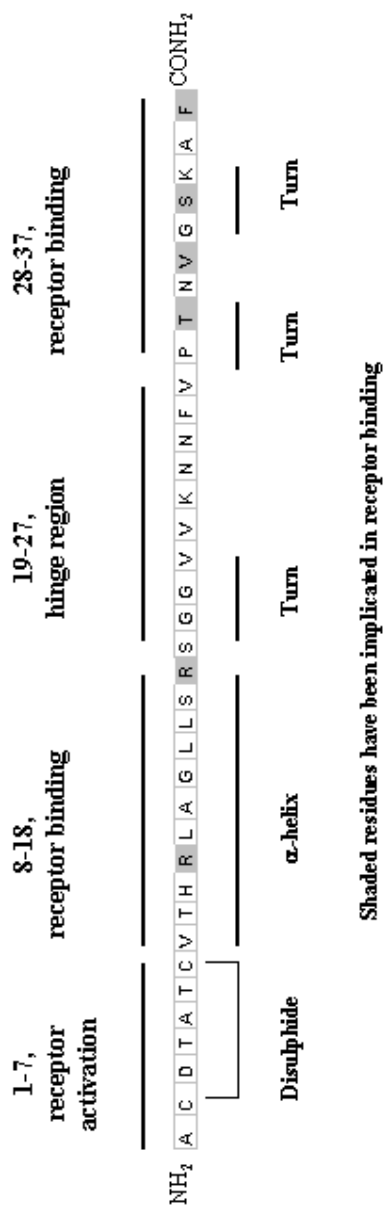
Shaded residues have been implicated in receptor binding

Figure 1.6. Structure of human α-CGRP

1.8.5   Distribution and localisation

CGRP and its receptors are widely distributed in the peripheral and central nervous systems as well as in the cardiovascular system (Wimalawansa, 2001). In the periphery, CGRP is abundantly present in the posterior horn cells. In primary sensory ganglia, CGRP is often co-stored with substance P (Lundberg *et al.*, 1985) and in motor neurons CGRP is co-stored with acetylcholine (Gibson *et al.*, 1988; Roa & Changeux, 1991). In most neurons, the α-and β-forms of CGRP co-exist, but the β-CGRP form is predominantly seen in the enteric nervous system (Mulderry *et al.*, 1988) and in the human pituitary gland (Jonas *et al.*, 1985).

In the cardiovascular system, CGRP-containing nerve fibres are more abundant around the arteries than around the veins (Bell & McDermott, 1996); in the arterial system, they are predominantly seen in the junction of the adventia and media (Wimalawansa, 2001). Moreover, CGRP-containing nerve fibres are seen more in the atria than in ventricles; within the atria, they are localised in the sino-atrial node, the atrio-ventricular node and the specilaised conduction system (Wimalawansa, 2001). In addition, the myocardium is less densely innervated than the epicardium, endocardium and pericardium (Wimalawansa, 2001). In the periphery, CGRP-containing nerve fibres are often associated with vascular smooth muscle such as: (i) most parts of gastrointestinal tract, including the excretory ducts of the parotid gland, over the epithelium of the fundic glands of stomach, endocrine cells of the duodenum and ileum and some myenteric ganglia; (ii) lung; (iii) thyroid gland (close to C cells); (iv) splenic vein and sinusoids; (v) human skin; and (vi) pituitary gland (Hagner *et al.*, 2002a; Hagner *et al.*, 2002b; Hagner *et al.*, 2002c). Moreover, i-CGRP has been found in the plasma of some patients with medullary thyroid and lung carcinoma and also in normal human beings (Girgis *et al.*, 1985; Takami *et al.*, 1985).

1.9   CGRP receptors

1.9.1   Classification

Based on functional studies (by using the C-terminal fragment of α-CGRP, α-CGRP$_{(8-37)}$ and linear CGRP analogues, [Cys(Acm)^{2,7}]- and [Cys(Et)^{2,7}]h-α-CGRP), CGRP receptors are classified into CGRP$_1$ and CGRP$_2$ subtypes (Table 1.3) (Conner *et al.*, 2002; Poyner *et al.*, 2002). Experimental evidence has shown that α-CGRP$_{(8-37)}$

behaved as a more potent antagonist on CGRP-induced responses in guinea pig atria (high affinity pA_2=7-8) than in those induced in rat vas deferens (lower affinity pA2 = 5.5-6.5) (Poyner *et al.*, 2002). In contrast, linear CGRP analogues, such as [Cys(Acm)^{2,7}]- and [Cys(Et)^{2,7}] hα-CGRP, have higher affinity for the rat *vas deferens* (EC_{50} = 234, 8.32 nM, respectively) than for the guinea pig atria (Conner *et al.*, 2002; Poyner *et al.*, 2002). Based on this evidence, it was proposed that the CGRP-induced responses in the guinea pig atria are mediated via $CGRP_1$ receptors and in the rat *vas deferens* by $CGRP_2$ receptors (Conner *et al.*, 2002; Poyner *et al.*, 2002). Furthermore, cell lines have now been characterised as uniquely enriched with the $CGRP_1$ (e.g. human SK-N-MC cells and rat L6 skeletal myocytes) and $CGRP_2$ receptors (e.g. COL-29 and HCA-7 colonic epithelium cells) (Juaneda *et al.*, 2000). Moreover, a potent and selective CGRP receptor antagonist, BIBN4096BS (Table 1.3), showed a 10-fold higher affinity for CGRP receptors in rat left atrium compared to than in rat *vas deferens*, supporting the existence of CGRP receptor subtypes in these two tissues. Interestingly, this study evidenced the presence of two CGRP-like receptor subtypes in rat vas deferens namely: (i) the $CGRP_2$ receptor; and (ii) a "novel" receptor that displays low efficacy for CGRP and that is selectively stimulated by [Cys(Et)^{2,7}]h-α-CGRP or amylin and which can be blocked with high affinity by BIBN4096BS (Wu *et al.*, 2000; Wu *et al.*, 2002). Moreover, BIBN4096BS also revealed additional functional differences between the actions of α-CGRP and β-CGRP in the pig left anterior descending coronary artery and in the cerebral basilar artery, indicating the existence of different CGRP receptor subtypes (Wu *et al.*, 2002). Notwithstanding the above findings, the molecular nature of both CGRP receptor subtypes remains far from clear and final demonstration must come from their respective cloning and the development of fully selective agonists and antagonists.

Table 1.3. Proposed classification of CGRP receptor subtypes (Juaneda *et al.*, 2000)

Parameter	Receptor subtype	
	CGRP$_1$	**CGRP$_2$**
Potency of endogenous homologues	CGRPα, CGRPβ >ADM>amylin	CGRPα, CGRPβ >ADM>amylin
Preferential agonist	None	Cys(Acm)^{2,7} hα-CGRP
Antagonist	*BIBN4096BS: pA$_2$ = 8-11; SK-N-MC cells *Compound 1: pA$_2$ = 7.7; SK-N-MC cells *SB-(+)-273779: pA$_2$ = 6.4; SK-N-MC cells **CGRP$_{(8-37)}$: pA$_2$ = 7-8	*BIBN4096BS: pA$_2$ = 6.5-7 **CGRP$_{(8-37)}$: pA$_2$ = 5.5-6.5
Second messenger	G$_s$ (cAMP production)	G$_s$
Prototypical bioassays	Atrium, pulmonary artery, spleen, SK-N-MC cells	Vas deferens, urinary bladder, liver, COL-29 and HCA-cells
Receptor associated proteins	CRLR, RAMP1, RCP	Unknown

Abbreviations: CGRP, calcitonin gene related peptide; COL-29 and HCA-7 cells, human colonic epithelium-derived cell line; CRLR, calcitonin receptor like receptor; RAMP, receptor activity modifying protein; RCP, receptor component protein; Cys(Acm)^{2,7}hα-CGRP, [acetamidomethyl-cysteine^{2,7}]CGRPα; G$_s$ (cAMP production), G-protein-coupled receptors that interact with G$_s$α to stimulate adenylate cyclase production; SK-N-MC cells, human neuroblastoma cell line; *, non-peptide CGRP receptor antagonist; **, peptide antagonist

1.9.2 Structure

The calcitonin receptor like receptor (CRLR), a G-protein coupled receptor (GPCR; family B) forms the basic receptor protein for CGRP and amylin receptors (Conner *et al.*, 2002; Poyner *et al.*, 2002). The evidence of CGRP functioning on the CRLR was based on the binding of CGRP and the accumulation of CGRP-dependent cAMP in a single subclone of HEK293 cells stably transfected with the human cloned receptor encoding cDNA (Aiyar *et al.*, 1996). Though CGRP and amylin bind with CRLR, the receptor specificity is being determined by a single transmembrane domain protein,

termed as the receptor activity modifying protein (RAMP) (McLatchie *et al.*, 1998; Mallee *et al.*, 2002). The RAMPs (150-177 aminoacids in size) are cleavable signal peptides, with a relatively large N-terminal extracellular domain, one transmembrane spanning domain and nine aminoacid intracellular C-terminal domains (Fitzsimmons *et al.*, 2003). The RAMPs have been localised in the endoplasmic reticulum and they are required to facilitate the intracellular translocation of the CRLR-maturing protein and its insertion into plasma membranes (McLatchie *et al.*, 1998; Hilairet *et al.*, 2001; Mallee *et al.*, 2002). Moreover, RAMPs can alter the pharmacology of the given CRLR by providing a mechanism whereby a cell could dynamically change its sensitivity

Figure 1.7. Schematic representation of $CGRP_1$ receptor showing interactions of RAMP1 protein with CRLR and a receptor component protein (RCP). This complex is tightly coupled to G_s to promote cAMP production.

from one receptor to another (McLatchie *et al.*, 1998; Mallee *et al.*, 2002). Three RAMPs have been identified in the human tissues namely, RAMP1, RAMP2 and RAMP3 (McLatchie *et al.*, 1998; Mallee *et al.*, 2002). Co-expression of CRLR with RAMP1 reveals CGRP receptors (Figure 1.7), whereas co-expression of CRLR with RAMP2 and RAMP3 forms adrenomedullin receptors (McLatchie *et al.*, 1998; Mallee *et al.*, 2002). The mechanism of action of RAMPs in CGRP/adrenomedullin binding is not clear, but in chimaeric RAMPs, it has been shown that the N-terminus of RAMP1 is the key determinant for CGRP binding, which could be due to the interaction of CRLR with N-terminus (Foord *et al.*, 1999). Similarly, in the human RAMP1, the extracellular domain of RAMP1 is sufficient for normal CRLR association and efficacy, while the specific sequences of the transmembrane domain contribute to CGRP affinity and specificity. The tail domain of RAMP1 is unnecessary for CRLR function (Fitzsimmons *et al.*, 2003). Moreover, in the human RAMP1, substitution of tryptophan at position 74 with lysine (as found in rat $RAMP_1$) confers low affinity and vice-versa suggesting important determinants for small molecule antagonists (Mallee *et al.*, 2002). In addition to RAMPs, the CGRP receptor complex requires another chaperone protein named as the receptor component protein (RCP) to function optimally (McLatchie *et al.*, 1998). The RCP is a 148-aminoacid,

intracellular protein that is required for G-protein-coupled signal transduction at the CGRP receptors (Prado *et al.*, 2002). The RCP is well expressed in CGRP responsive tissues and RCP expression correlates to the biological efficacy of CGRP *in vivo* (Evans *et al.*, 2000).

The structure of $CGRP_2$ receptor subtypes is unclear and little has been done so far to characterise the structural requirements for the CGRP binding to $CGRP_2$ receptors (Poyner & Marshall, 2001). The linear CGRP analogue $Cys(Acm)^{2,7}h\alpha$-CGRP has been used to classify $CGRP_2$ receptor subtypes, but an agonist may not definitively characterise a receptor (Poyner & Marshall, 2001). The *mRNA* for the CRLR receptor is present in the rat vas deferens as well as in other tissues showing pharmacological properties similar to those of the putative $CGRP_2$ receptor subtypes, e.g. the porcine coronary artery (Conner *et al.*, 2002; Hay *et al.*, 2002; Poyner *et al.*, 2002). Moreover, other studies have demonstrated that, in general, the affinity of ligands for $CGRP_1$ receptors is higher than for $CGRP_2$ receptors, which could be attributable to tissue factors such as proteases (Rorabaugh *et al.*, 2001) and/or to a deficiency of one or more ligand-binding contacts (Conner *et al.*, 2002). However, further work is necessary to confirm $CGRP_2$ receptors structure, activity and effects in the tissues.

1.9.3   Distribution and binding

CENTRAL NERVOUS SYSTEM

The distribution of CGRP receptors has been well documented and they generally match with CGRP binding studies (Wimalawansa, 1996). In the CNS, both high and low binding sites for CGRP have been reported (Wimalawansa, 1996; Morara *et al.*, 2000; Segond von Banchet *et al.*, 2002). For example, moderate to high density of CGRP receptors was found in the piriform-insular cortex complex, while low to moderate receptors were seen in the medial preoptic area (Wimalawansa, 1996). In contrast, very high levels of CGRP receptors are seen in the solitary, vagus, hypoglossus, dorsal medullary reticular nuclei and in the area postrema (Wimalawansa, 1996). The highest density of CGRP receptors is found in the cerebellum and dorsal spinal cord (Wimalawansa, 1996). In cerebellum, its molecular layer contains the highest density of CGRP receptors, whilst the Purkinje cell layer contains a moderate number; in contrast, its granular layer is devoid of CGRP

receptors (Morara *et al.*, 1998; Morara *et al.*, 2000; Ueda *et al.*, 2001). In dorsal root ganglion and other neurons, CGRP receptors co-exist with receptors for other neurotransmitters and neuromodulators such as, substance P, noradrenaline, neuropeptide Y, vasoactive intestinal peptide, histidine (van Rossum *et al.*, 1997; Ohtori *et al.*, 2002). Moreover, in the nonadrenergic and noncholinergic (NANC) fibres and in the coronary vessels, CGRP receptors co-exist with receptors for tachykinins (Ursell *et al.*, 1991) and substance-P, respectively (Wiesenfeld-Hallin *et al.*, 1984).

CARDIOVASCULAR SYSTEM

The highest density of CGRP binding sites is present in the heart and in the blood vessels (intima and media layers) (Sigrist *et al.*, 1986; Wimalawansa, 2001). Moreover, in the heart, high affinity binding sites for CGRP are found in the atrial and ventricular preparations (Wimalawansa, 2001). Regardless of the species, the density of the CGRP binding sites in atria invariably exceeds that of ventricles (Chang *et al.*, 2001). Autoradiographic studies in the hearts of rats (Chang *et al.*, 2001), guinea pigs and humans (Coupe *et al.*, 1990; Hasbak *et al.*, 2003) have shown the highest density of CGRP binding sites in the coronary arteries, coronary veins and in the heart valves, while a lower density is found in the coronary arterioles and endocardium (Wimalawansa, 2001).

CGRP receptors are also abundantly present in the thyroid gland, gastrointestinal tract, parotid gland, adrenals, pituitary, exocrine pancreas, kidneys, bones, skin and skeletal muscles (Wimalawansa, 1996; Hagner *et al.*, 2002a; Hagner *et al.*, 2002b; Hagner *et al.*, 2002c; Irie *et al.*, 2002; Rossi *et al.*, 2003).

1.9.4   Signal transduction mechanisms

The CGRP-induced vascular responses are mediated by both endothelium-dependent and endothelium-independent mechanisms (Marshall, 1992; Wimalawansa, 1996) (Figure 1.8). In many blood vessels including the thoracic aorta, renal, pulmonary and cerebral arteries, the endothelium is absolutely required for CGRP-induced vasodilatory responses (Wimalawansa, 1996). This endothelium-dependent pathway begins with CGRP binding to its receptors in the endothelial cells, activating adenylyl cyclase thereby increasing cAMP levels. This increase in cAMP levels may then activate the enzyme nitric oxide synthase (NOS), leading to an increase level of nitric

oxide (NO) (Lincoln, 1989). However, CGRP may also elevate NO directly without the involvement of adenlycyclase. This elevated NO acts on the smooth muscle cells by activating guanylate-cyclase with an ensuing production of cGMP leading to smooth muscle relaxation (Wimalawansa, 1996).

In the endothelium-independent pathway, CGRP bypasses the endothelium and directly binds to CGRP receptors on the smooth muscle cells, activating adenylyl cyclase; this, in turn, will increase cAMP levels leading to vascular relaxation (Wimalawansa, 1996). This endothelium-independent vasorelaxation has been demonstrated in several arteries, including the (i) feline cerebral artery; (ii) porcine splenic artery; (iii) human skeletal muscle artery; (iv) rabbit mesenteric artery; (v) rat gastric, splenic and hepatic arteries; and (vi) guinea pig pulmonary artery (Wimalawansa, 1996; van Rossum *et al.*, 1997). Interestingly, blood vessels such as the rat basilar and superior mesenteric arteries require both endothelium-dependent and endothelium-independent mediated mechanisms. CGRP also act indirectly by stimulating protein kinase A that activates K^+_{ATP} channels (in rabbit arterial smooth muscle) (van Rossum *et al.*, 1997).

Evidently, CGRP-induced responses involve multiple second messengers, including cAMP, nitric oxide-cGMP and K^+ channels (van Rossum *et al.*, 1997). Notwithstanding, irrespective of second messengers involved, the final common pathway for CGRP-induced vasorelaxation depends ultimately upon the decrease in intracellular calcium (Wimalawansa, 1996).
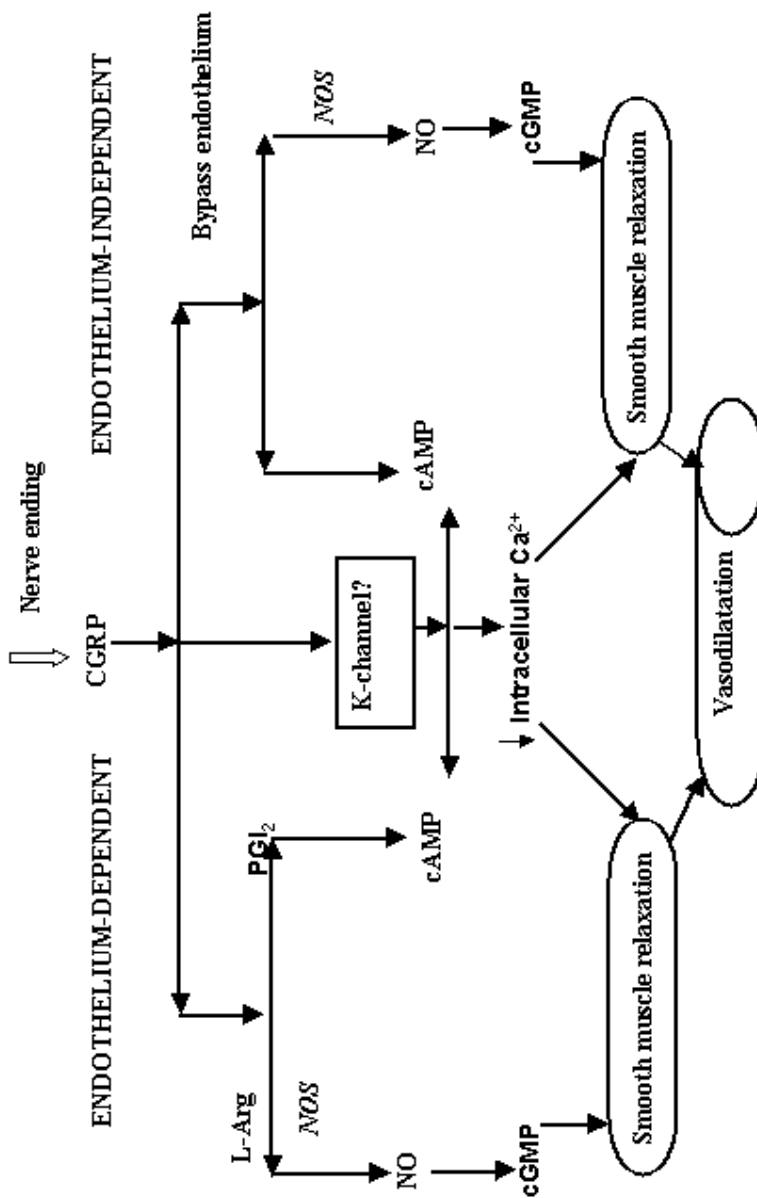
Figure 1.8. Signal transduction mechanisms of CGRP receptors

1.10   CGRP peptide Assay

The CGRP levels in the circulation are rather low (average 2 to 35 pM); its half-life is about 10-12 min.   The target cells contain cell surface enzymes (neutral endo-peptidase) that cleave CGRP (Wimalawansa, 1996).   The plasma CGRP levels can be measured by several *in vitro* assays, namely: (i) radioreceptor assay (RRA) and (ii) radioimmunoassay (RIA).   The bioassays used to measure the potency of CGRP includes: (i) cAMP generation, (ii) rat aortic strip relaxation assay, (iii) the inotrophic and chronotrophic effects on the rat heart, and (iv) the assay for cardiovascular functions, such as change in the mean blood pressure (Wimalawansa, 1996).

Electrical or chemical stimulation (capsaicin) of sensory nerves ensue the release of endogenous CGRP (Goadsby *et al.*, 1990; Kapoor *et al.*, 2003a).   Moreover, the administration of capsaicin during the neonatal period of the rats will destroy CGRP containing sensory C fibres; this indicates that the circulating CGRP levels is due to its overspill from the perivascular nerves (Medeiros *et al.*, 2003).   Furthermore, receptors for bradykinin and histamine (present on the sensory nerves) modulate the release of CGRP (de Hoon *et al.*, 2003; Sato *et al.*, 2003).

1.11   Physiological functions of CGRP

CARDIOVASCULAR SYSTEM

The wide distribution of CGRP and its receptors in the cardiac tissue (sinoatrial node, coronary arteries, atrial and ventricular musculature) correlates well with its postulated functions, such as increases in: (i) heart rate, (ii) force of contraction, (iii) coronary blood flow, and (iv) microvascular permeabilty.   Moreover, several studies have demonstrated that these chronotropic and inotropic responses are mediated via CGRP receptors (Bell & McDermott, 1996; Kaygisiz *et al.*, 2003).   Furthermore, CGRP is believed to play a notable role in the regulation of the vascular tone and angiogenesis (Bell & McDermott, 1996).

Infusion of CGRP decreases the perfusion pressure in isolated hearts, indicating its vasodilating action on coronary vasculature (Bell & McDermott, 1996; Hasbak *et al.*, 2003).   In addition, CGRP: (i) has a cardioprotective effect through mediation of the preconditioning induced by brief ischaemia (Yallampalli *et al.*,

2002); and (ii) directly affects the capacitance blood vessels thereby producing peripheral vasodilatation (Wimalawansa, 1996).

CGRP receptors are abundantly present on renal blood vessels (Wimalawansa & MacIntyre, 1988), where they have several functions, namely: (i) to increase the renal blood flow and glomerular filtration rate; (ii) to relax the afferent arterioles in the glomeruli; (iii) to increase the renin production; and (iv) to stimulate the release of atrial naturetic peptide (Wimalawansa, 1996).

CENTRAL NERVOUS SYSTEM

In the CNS, CGRP plays an important role in various functions including the motor, sensory and integrative systems (van Rossum *et al.*, 1997). Moreover, CGRP modulates several senses including audition, olfaction, vision, feeding and behavioural effects (van Rossum *et al.*, 1997). The wide distributions of i-CGRP in the parabrachial area (a relay centre for autonomic-related functions) indicate its role in regulating the cardiovascular, respiratory and sleep functions (van Rossum *et al.*, 1997). Consistent with this anatomical distribution, microinjection of CGRP into the central nucleus of amygdala increased the heart rate and mean arterial blood pressure; this is due to catecholamines release following sympathetic stimulation via CGRP receptors in the CNS (Kuo *et al.*, 1994; Oh-hashi *et al.*, 2001).

The CGRP receptors are widely distributed in the parabrachial nucleus and in the nucleus tractus solitarii; this suggests that CGRP receptors may be involved in relaying the visceral sensory information from the vagus and glossopharyngeal nerves (Sykes *et al.*, 1994). Moreover, the association of CGRP receptors with the vagus and glassopharyngeal nerves may be responsible for the CGRP-induced responses on the cardiovascular functions including baroreceptor reflexes (Sykes *et al.*, 1994; van Rossum *et al.*, 1997).

Amongst other effects, CGRP seems to regulate: (i) growth hormone secretion; (ii) hyperthermia; (iii) catalepsy; (iv) motor activity; and (v) nociceptive responses (van Rossum *et al.*, 1997). Furthermore, CGRP potentiates excitatory actions by enhancing the release of substance P as well as of excitatory aminoacids from the primary afferent fibres (Oku *et al.*, 1987; Kangrga *et al.*, 1990), hence the possibility of leading to an increased activity of these transmitters.

In efferent nerve fibres, CGRP co-exists with acetylcholine-containing neurons. These nerve fibres innervate the motor end plate, where CGRP modulates the release of acetylcholine and potentiates the response to acetylcholine (Rossi *et al.*, 2003). Moreover, CGRP also acts as a neurotrophic factor by increasing the synthesis of acetylcholine receptors (Rossi *et al.*, 2003).

OTHER FUNCTIONS

Additional biological functions mediated by CGRP include: (i) regulation of the pituitary hormone secretion; (ii) release of pancreatic enzymes; (iii) control of gastric acid secretion; (iv) thermoregulation; (v) decrease in food intake; (vi) antagonism some actions of insulin; and (vii) growth-factor like functions (Wimalawansa, 1996).

In bones, CGRP induces its effect via calcitonin receptors, thereby producing: (i) hypocalcemia; (ii) proliferation of osteoclasts; and (iii) inhibition of both basal and stimulated resorption of the intact bone (Villa *et al.*, 2003). Moreover, several studies have shown that CGRP containing nerve fibres are widely distributed in bone tissues (periosteum and bone marrow); this apparent distribution suggests that CGRP may be involved in bone remodelling (Irie *et al.*, 2002).

Several lines of evidence have shown that an increase in plasma CGRP levels is produced during pregnancy, menstruation and following oral contraception. This significant increase in plasma CGRP levels may be specific (17β-estradiol-mediated increase of CGRP synthesis) and/or due to a compensatory mechanism evoked by an increase in blood volume. Moreover, CGRP inhibits spontaneous contractions in the uterus and fallopian tubes; therefore, any defect in the CGRP synthesis might lead to complications during pregnancy (Gangula *et al.*, 2002; Yallampalli *et al.*, 2002).

CGRP increases microvascular permeability, thereby resulting in: (i) inflammatory hyperaemia; (ii) neutrophil accumulation; and (iii) localised oedema (Brain & Williams, 1985; Brain *et al.*, 1985; Brain *et al.*, 1986). Moreover, in a neurogenic inflammation model, CGRP produces vascular leakage; this response to CGRP was completely attenuated by CGRP$_{(8-37)}$ (Escott & Brain, 1993). In contrast, CGRP also mediates antiinflammatory effects, such as maintaining mucosal blood flow in the gastric mucosal injury model (Gennari & Fischer, 1985; van Rossum *et al.*, 1997).

CGRP may induce angiogenesis by promoting migration of the endothelial cells during physiological and pathological conditions such as ischaemia, inflammation and wound healing (Bell & McDermott, 1996; Ackermann *et al.*, 2002).

1.12 Therapeutic potentials of CGRP receptor ligands

1.12.1 CGRP agonists

Several studies have shown that CGRP receptor agonists are under consideration for many clinical conditions (Wimalawansa, 1996). Because of its potent vasodilatory effects, it is now being considered that CGRP receptor agonists may be used in vascular diseases, such as coronary heart disease and myocardial ischaemia (CGRP relieves arterial vasospasm) (Wimalawansa, 1996). Infusions of CGRP: (i) improve exercise tolerance; (ii) increase the diameter of epicardial coronary artery (at the site of atheromatous stenoses); and (iii) delay the onset of myocardial ischaemia (Bell & McDermott, 1996). Moreover, in patients with congestive cardiac failure, CGRP increases cardiac output and decreases blood pressure, without altering the heart rate (Bell & McDermott, 1996).

It has been suggested that during myocardial infarction, there is: (i) an upregulation of CGRP receptors in sympathetic ganglia; and (ii) an increase in plasma CGRP levels. These findings indicate that CGRP may be involved in the regulation of heart ischaemia (Bell & McDermott, 1996; Roudenok & Schmitt, 2001). Moreover, infusions of CGRP reduce markers of myocardial ischaemia, such as creatinine phosphokinase and glutamine-oxaloacetic transaminase (Bell & McDermott, 1996; Roudenok & Schmitt, 2001). Furthermore, CGRP agonists can be used as antiarrhythmic agents, because they reduce the degree of arterioventricular blockade and protect against ventricular fibrillation (Bell & McDermott, 1996).

The clinical application of CGRP agonists for hypertension is of great interest; as an experimental rat model for hypertension demonstrated a significant decrease in: (i) plasma CGRP levels; (ii) CGRP contents in perivascular nerves; and (iii) the vascular sensitivity to CGRP (Wimalawansa, 2001). Moreover, infusions of β-CGRP in the hypertensive patients significantly decrease the blood pressure (Wimalawansa, 2001).

Other clinical applications of CGRP agonists are: (i) Raynaud's syndrome; (ii) peripheral vascular diseases (thrombo-embolism or diabetic vascular disease);

(iii) subarchanoid haemorrhage; (iv) nerve and neuromuscular regeneration; (v) erectile dysfunction; (vi) pulmonary hypertension; (vii) pre-eclamptic toxaemia and preterm labour; and (viii) venous stasis ulcer (Bivalacqua *et al.*, 2001; Wimalawansa, 2001; Ackermann *et al.*, 2002; Ellington *et al.*, 2002; Knerr *et al.*, 2002; Qing & Keith, 2003).

In view of wide variety of effects produced by CGRP, its systemic administration would produce an array of side effects. Therefore, oral and long acting CGRP-mimetics may be useful in disorders where CGRP administration has been shown to be beneficial. Some known CGRP mimetics are capsaicin/vanilloid receptor agonists and gene transfer of an adenoviral vector that encodes prepro-CGRP (Doggrell, 2001). Examples include, capsiate (a capsaicin-like ingredient of a non-pungent cultivar of red pepper,), anandamide, etc.

1.12.2   CGRP receptor antagonists

Several lines of evidence have shown that an inappropriate release of CGRP is a potential causative factor in several diseases, including: (i) migraine (discussed below); (ii) inflammation (as meningitis); (iii) cardiogenic shock associated with sepsis; and (iv) thermal injury (Wimalawansa, 1996; Hoffmann *et al.*, 2002). Moreover, other studies have demonstrated that CGRP receptor antagonists can be used in treating insulin resistant type II diabetes mellitus. (Miyamoto *et al.*, 2001).

The role of CGRP in inflammation, pain and nociception is well established; CGRP potentiates oedema formation by stimulating mediators of vascular permeability and chemotactic factors leading to neutrophil accumulation (Wimalawansa, 1996; de Hoon *et al.*, 2003; Jarvikallio *et al.*, 2003; Kawasaki *et al.*, 2003; Low & Merikangas, 2003; Ma *et al.*, 2003; Sato *et al.*, 2003). Other findings in arthritic rats suggest a marked increase in i-CGRP in the dorsal horn (Wimalawansa, 1996); this indicates that CGRP may be involved in the nociception associated with cutaneous inflammation (Sun *et al.*, 2003). Furthermore, CGRP and substance P containing nerve fibres are abundantly seen in atopic dermatitis and nummular eczema (Jarvikallio *et al.*, 2003). Therefore, CGRP receptor antagonists may dampen an inflammatory response, neurogenic inflammation and/or pain transmission (de Hoon *et al.*, 2003; Jarvikallio *et al.*, 2003; Kawasaki *et al.*, 2003; Low & Merikangas, 2003; Ma *et al.*, 2003; Sato *et al.*, 2003).

1.13 CGRP and migraine; potential targets for migraine therapy

Several studies have shown that the stimulation of trigeminal ganglia/sensory nerves releases CGRP, which dilates cranial blood vessels and stimulates sensory nerve transmission (Goadsby *et al.*, 2002b; Edvinsson, 2003). Interestingly, during the headache phase of migraine, plasma concentrations of CGRP, but not other neuropeptides, are elevated. Hence, trigeminal CGRP release is considered as a marker for migraine that can be measured in a venous blood sample; the decrease in this marker seems to be highly predictive of antimigraine activity in humans (Goadsby *et al.*, 2002b; Edvinsson, 2003; Hasbak *et al.*, 2003). In line with this finding, CGRP is believed to play a central role in migraine pathophysiology. Therefore, it is reasonable to assume that a substance capable of inhibiting trigeminal CGRP release or antagonising vascular CGRP receptors may be an effective antimigraine strategy (Goadsby *et al.*, 2002b; Edvinsson, 2003; Hasbak *et al.*, 2003). Either strategy would ultimately result in the prevention of cranial vasodilatation, as clearly demonstrated for essentially all acute antimigraine agents, such as the triptans and ergot derivatives (Villalón *et al.*, 2002). In this respect, it is noteworthy that the acute antimigraine agents have been reported to abort migraine attacks by at least two main mechanisms, namely: (i) constriction of dilated cranial arteries and arteriovenous anastomoses via the stimulation of 5-HT$_{1B}$ receptor (De Vries *et al.*, 1999b; Villalón *et al.*, 2002); and (ii) inhibition of CGRP release as well as of nociceptive transmission on peripheral and central trigeminal sensory nerves via 5-HT$_{1B/1D}$ receptors (Bigal *et al.*, 2002; Goadsby *et al.*, 2002b; Tepper *et al.*, 2002)

1.13.1 Inhibition of CGRP release

Cerebral blood vessels are innervated by sensory nerves that store several neuropeptides of which CGRP is the most abundant (Williamson & Hargreaves, 2001). As discussed above, it has been proposed that triptans alleviate migraine by: normalising the elevated plasma CGRP levels (Goadsby *et al.*, 2002b). Though triptans represent a significant advance in migraine therapy, they are ineffective in some patients and have limitations due to their potential side effects (Maassen VanDenBrink *et al.*, 1999; MaassenVanDenBrink *et al.*, 2000a). Therefore, the crucial improvement in antimigraine therapy would seem to be the development of an antimigraine agent with no (cardio) vascular side effects (Goadsby *et al.*, 2002b), but

still capable of inhibiting the trigeminal release of CGRP. In this context, selective agonists at 5-HT$_{1D}$ (e.g. PNU-109291) (Ennis *et al.*, 1998) and 5-ht$_{1F}$ (e.g. LY344864, LY334370) (Johnson *et al.*, 1997; Phebus *et al.*, 1997; Ramadan *et al.*, 2003) receptors are devoid of contractile effects on coronary and cerebral blood vessels (Bouchelet *et al.*, 2000; Villalón *et al.*, 2002). However, PNU-142633 proved to be ineffective in the acute treatment of migraine (Gómez-Mancilla *et al.*, 2001), whilst LY334370 did show some efficacy when used in doses, which interact with 5-HT$_{1B}$ receptors (Ramadan *et al.*, 2003).

On the basis of the above, the potential role of α_2-adrenoceptor subtypes and adenosine A$_1$ receptors in inhibiting the CGRP release may prove vital in developing a potent antimigraine compound (Goadsby *et al.*, 2002a; Willems *et al.*, 2003). Indeed, several experimental studies have shown that a selective adenosine A$_1$ receptor agonist, GR79236, inhibits trigeminal nociception and CGRP release (Goadsby *et al.*, 2002a; Giffin *et al.*, 2003). Other inhibitors of CGRP release include antagonists at capsaicin/vanilloid receptors and CGRP receptor (see below) (Doggrell, 2001).

1.13.2   Antagonism of CGRP receptors

Truncated fragments of CGRP, such as CGRP$_{(8-37)}$, function as CGRP receptor antagonists (Juaneda *et al.*, 2000). However, CGRP$_{(8-37)}$ proved ineffective in migraine treatment due to its low potency and shorter half-life (Chiba *et al.*, 1989; Rist *et al.*, 1998). An important breakthrough in the field of CGRP is the development of potent CGRP receptor antagonists (Figure 1.9), namely: (i) BIBN4096BS (Doods *et al.*, 2000); (ii) compound 1 (Hasbak *et al.*, 2001; Hasbak *et al.*, 2003); and (iii) SB-(+)-273779 (Aiyar *et al.*, 2001). Both BIBN4096BS and compound 1 are 2-3 log units more potent in human tissues than in those of other animals (Edvinsson, 2003; Hasbak *et al.*, 2003). BIBN4096BS demonstrates extremely high affinity for human CGRP receptors expressed in SK-NM-C cells (K$_i$ = 14.4±6.3 pM) (Hay *et al.*, 2002; Schindler & Doods, 2002; Wu *et al.*, 2002). Several studies have shown that BIBN4096BS clearly attenuates: (i) the vasodilatation induced by trigeminal stimulation in marmosets (Doods *et al.*, 2000); and (ii) capsaicin and α-CGRP-induced porcine carotid vasodilator responses (Kapoor *et al.*, 2003a; Kapoor *et al.*, 2003b). Moreover, in human cerebral vessels, BIBN4096BS

behaves as a potent antagonist than in peripheral arteries (Edvinsson, 2003; Hasbak *et al.*, 2003).

BIBN4096BS

Compond 1
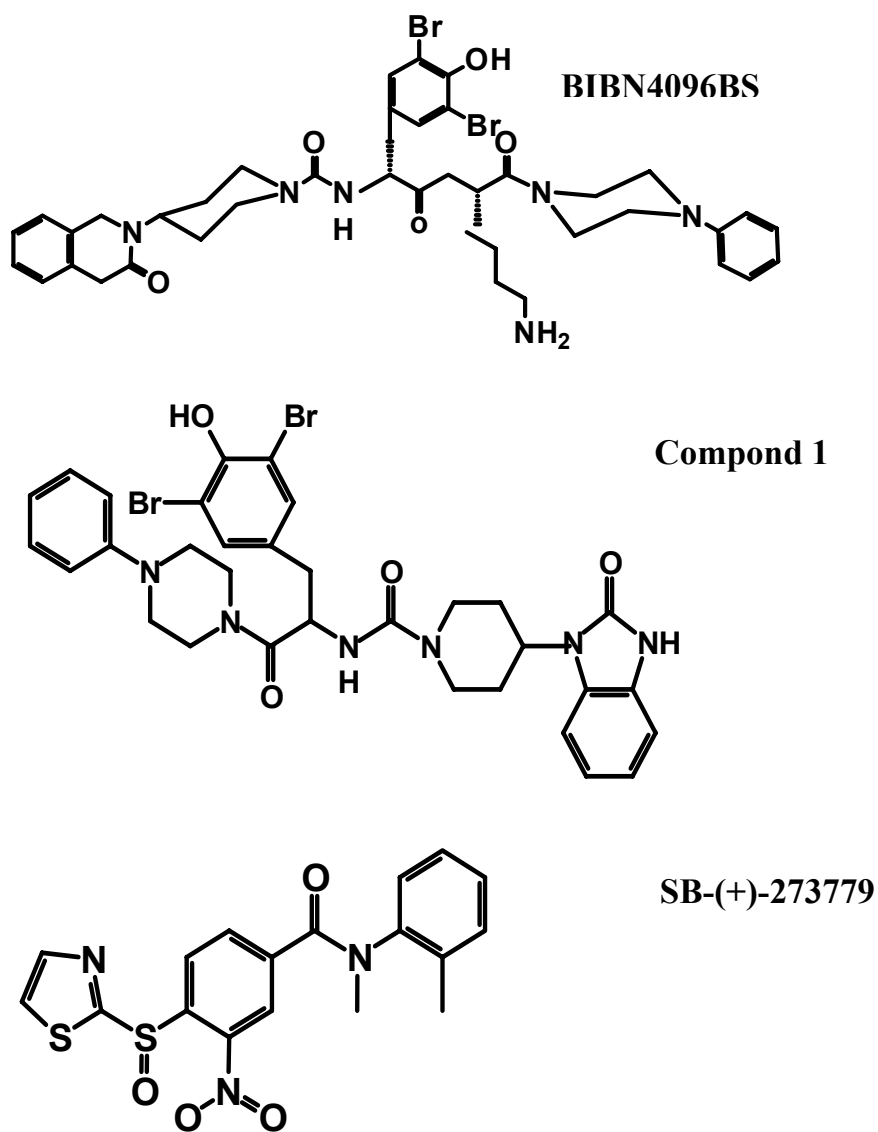
SB-(+)-273779

Figure 1.9. Chemical structure of BIBN4096BS, compound 1 and SB-(+)-273779

Similar to BIBN4096BS, compound 1: (i) displaced ^{125}I-CGRP from SK-N-MC cells; (ii) antagonised the CGRP-induced increase in cAMP production with pA_2 values of ~8 nM; and (iii) produced a parallel rightward shift of the concentration-response curve of CGRP in human cerebral arteries with a pA_2 value of 10nM (Hasbak *et al.*, 2001; Edvinsson, 2003; Hasbak *et al.*, 2003).

A third CGRP$_1$ receptor antagonist, SB-(+)-273779, inhibited the CGRP binding to SK-N-MC cells and reduced CGRP-induced adenylate cyclase activity (Aiyar *et al.*, 2001). Moreover, SB-(+)-273779 has no significant affinity for other receptors including those for calcitonin, endothelin, angiotensin-II and catecholamines. Therefore, SB-(+)-273779 can be a useful tool for studying CGRP-mediated functional responses in several experimental models as it does not appear as selective for human CGRP receptors as compared to BIBN4096BS and compound 1 (Edvinsson, 2003; Hasbak *et al.*, 2003). Table 1.4 shows the apparent pK$_B$ values for various CGRP receptor antagonists on different cell lines and different tissues.

In conclusion, the potential correlation between CGRP release and migraine has pointed out a need for a compound that counteracts CGRP-induced responses. Preliminary clinical results with BIBN4096BS given intravenously have shown that the compound is effective in treating migraine with no significant side effects (Edvinsson, 2003; Olesen *et al.*, 2003a). It remains to be seen if it is equally effective by the oral route and whether its efficacy is comparable to that of triptans. Moreover, there is no direct evidence on contractile effects of this CGRP receptor antagonist, which may provide an advantage over triptans, provided that they have similar efficacy (Edvinsson, 2003).

1.14   Aims of this thesis

- To study in anaesthetised vagosympathectomised pigs, the effects of BIBN4096BS (a potent and selective CGRP receptor antagonist) and sumatriptan (a 5-HT$_{1B/D}$ receptor agonist with established antimigraine properties) on capsaicin-induced: (i) carotid haemodynamic responses; and (ii) plasma CGRP concentrations.

- To investigate, in anaesthetised vagosympathectomised pigs, the effects of BIBN4096BS on: (i) α-CGRP induced changes in carotid haemodynamics; and (ii) cardiac output distribution, in order to establish BIBN4096BS cardiovascular safety.

- To examine the cardiovascular distribution of CGRP receptors in anaesthetised rats by investigating the effects of BIBN4096BS on α-CGRP-induced changes in cardiac output distribution and regional haemodynamics.

CHAPTER 2

Effects of the CGRP receptor antagonist BIBN4096BS on capsaicin-induced carotid haemodynamic changes in anaesthetised pigs

2 Effects of the CGRP receptor antagonist BIBN4096BS on capsaicin-induced carotid haemodynamic changes in anaesthetised pigs

Abstract: Calcitonin gene-related peptide (CGRP), a potent vasodilator released from capsaicin-sensitive trigeminal sensory nerves, seems to be involved in the pathogenesis of migraine. Hence, CGRP receptor antagonists may serve as a novel treatment for migraine. This study was therefore designed to investigate the effects of BIBN4096BS (100, 300 and 1000 $\mu g\,kg^{-1}$, i.v.), a potent and selective CGRP receptor antagonist, on capsaicin-induced carotid haemodynamic changes in anaesthetised pigs. Both vagosympathetic trunks were cut and phenylephrine was infused into the carotid artery (i.c.) to support carotid vascular tone. Infusions of capsaicin (0.3, 1, 3 and 10 $\mu g\,kg^{-1}\,min^{-1}$, i.c.) did not alter heart rate, but dose-dependently increased mean arterial blood pressure. This moderate hypertensive effect was not modified by BIBN4096BS. Capsaicin infusion (10 $\mu g\,kg^{-1}\,min^{-1}$, i.c.) increased total carotid, arteriovenous anastomotic and tissue blood flows and conductances as well as carotid pulsations, but decreased the difference between arterial and jugular venous oxygen saturations. These responses to capsaicin were dose-dependently blocked by BIBN4096BS. Capsaicin infusion (10 $\mu g\,kg^{-1}\,min^{-1}$, i.c.) more than doubled jugular venous plasma concentration of CGRP. This effect was not blocked, but rather increased, by BIBN4096BS. The above results show that BIBN4096BS behaves as a potent antagonist of capsaicin-induced carotid haemodynamic changes that are mediated via the release of CGRP. Therefore, this compound may prove effective in the treatment of migraine.

2.1 Introduction

Although a complete understanding of the pathogenesis of migraine remains elusive thus far, there seems little doubt that dilatation of cranial blood vessels, including carotid arteriovenous anastomoses, is involved in the headache phase (De Vries *et al.*, 1999a). Moreover, evidence is accumulating that a release of vasoactive neuropeptides from the trigeminal sensory nerves may be an important factor in the genesis of migraine (Goadsby *et al.*, 2002b). In this respect, a high circulating plasma concentration of calcitonin gene related peptide (CGRP) has been demonstrated during migraine headache (Goadsby *et al.*, 1990) and these concentrations can be normalised by triptans in parallel with alleviation of headache (Goadsby *et al.*, 1990; Ashina *et al.*, 2000). Indeed, CGRP is widely distributed in the body, including the central and peripheral parts of the trigeminovascular system (Brain *et al.*, 1985; van Rossum *et al.*, 1997; Juaneda *et al.*, 2000; Poyner & Marshall, 2001), where it is co-localised with substance P, neurokinin A and/or 5-HT$_{1D}$ receptors (Gulbenkian *et*

al., 1995; Gulbenkian *et al.*, 2001; Smith *et al.*, 2002). CGRP can mediate neurogenic dilatation of cranial blood vessels as well as sensory nerve transmission between the first and second order afferent input from these vessels during migraine headache (Gulbenkian *et al.*, 2001; Williamson & Hargreaves, 2001; Goadsby *et al.*, 2002b; Smith *et al.*, 2002). Thus, it follows that inhibition of CGRP-mediated cranial vasodilatation and sensory nerve transmission with a potent and selective CGRP receptor antagonist may prove a novel strategy in treating migraine.

The recent discovery of a di-peptide CGRP receptor antagonist BIBN4096BS (1-piperidinecarboxamide, N-[2-[[5-amino-1[[4-(4-pyridinyl)-1-piperazinyl]carbonyl] pentyl]amino]-1-[(3,5-dibromo-4-hydroxyphenyl)methyl]-2-oxoethyl]-4-(1,4 dihydro-2-oxo-3(2H)-quinazolinyl)-, (Doods *et al.*, 2000; Doods, 2001) represents a significant advance in exploring the pathophysiological role of CGRP in migraine. BIBN4096BS displays a very high affinity for human CGRP receptors (Doods *et al.*, 2000; Wu *et al.*, 2000; Edvinsson *et al.*, 2002; Moreno *et al.*, 2002). This compound is undergoing clinical trials for aborting migraine headache and the clinical results are awaited with great interest.

Using an animal model that seems to be predictive of antimigraine activity (Spierings & Saxena, 1980; Villalón & Terrón, 1994; Saxena, 1995; Saxena *et al.*, 1998; De Vries *et al.*, 1999a; Tfelt-Hansen *et al.*, 2000), the present study in anaesthetised pigs was designed (i) to investigate the effects of capsaicin (pungent substance in red chilli pepper), which releases neuropeptides, including CGRP (Alving *et al.*, 1991; Jansen-Olesen *et al.*, 1996; Szallasi & Blumberg, 1999; Eltorp *et al.*, 2000), on systemic and carotid haemodynamics, and (ii) to establish if BIBN4096BS is able to attenuate the responses induced by capsaicin. A preliminary account of this investigation was presented at the XIVth World Congress of Pharmacology (Kapoor *et al.*, 2002).

2.2 Materials and methods

2.2.1 General

After an overnight fast, a total of 22 pigs (Yorkshire x Landrace, females, 10-14 kg; n=11 each for vehicle and BIBN4096BS) were sedated with azaperone (120 mg, i.m.) and midazolam hydrochloride (10 mg, i.m.) and then anaesthetised with sodium pentobarbital (600 mg, i.v.). After tracheal intubation, the animals were connected to

a respirator (BEAR 2E, BeMeds AG, Baar, Switzerland) for intermittent positive pressure ventilation with a mixture of room air and oxygen. Respiratory rate, tidal volume and oxygen supply were adjusted to keep arterial blood gas values within physiological limits (pH: 7.35-7.48; pCO_2: 35-48 mmHg; pO_2: 100-120 mmHg). Anaesthesia was maintained with a continuous i.v. infusion of sodium pentobarbital (12-20 mg kg^{-1} h^{-1}). This anaesthetic regimen, together with bilateral vagosympathectomy (see below), increases heart rate and markedly dilates carotid arterioles and arteriovenous anastomoses due to a loss of parasympathetic and sympathetic tone, respectively. Consequently, carotid blood flow, particularly its arteriovenous anastomotic fraction, is considerably higher in these pigs than in conscious or thiopental-anaesthetised pigs (Den Boer *et al.*, 1993).

Heart rate was measured with a tachograph (CRW, Erasmus University, Rotterdam, The Netherlands) triggered by electrocardiogram signals. Both common carotid arteries were dissected free and the accompanying vagosympathetic trunks were cut between two ligatures to prevent any possible influence via baroreceptor reflexes on the carotid vascular responses produced by capsaicin. Pulsatile and mean carotid blood flows were measured in the right common carotid artery with a flow probe (internal diameter: 2.5 mm) connected to a sine-wave electromagnetic flow meter (Transflow 601-system, Skalar, Delft, The Netherlands). The amplitude of carotid blood flow signals provided an index of carotid flow pulse. Subsequently, three hub-less needles, connected to a polyethylene tube, were inserted into the right common carotid artery for the administration of capsaicin, radioactive microspheres and the α_1-adrenoceptor agonist phenylephrine. The use of phenylephrine is necessitated by the fact that the carotid arterioles and arteriovenous anastomoses are already in a dilated state under the present anaesthetic regime (Den Boer *et al.*, 1993) and, therefore, to study the effects of vasodilator agents (in the present case capsaicin) one has to constrict them first. As described earlier (Willems *et al.*, 1999), phenylephrine decreases total carotid conductance exclusively by constricting carotid arteriovenous anastomoses, which results in an increase in the difference between arterial and jugular venous oxygen saturations (A-V SO_2 difference) (Saxena, 1987).

Lastly, catheters were placed in the right external jugular vein for the withdrawal of venous blood samples to measure blood gases (ABL-510; Radiometer, Copenhagen, Denmark) and plasma concentrations of CGRP (see below), inferior

vena cava (via the left femoral vein) for the administration of the vehicle or BIBN4096BS and aortic arch (via the left femoral artery) for the measurement of arterial blood pressure (Combitrans disposable pressure transducer; Braun, Melsungen, Germany) as well as withdrawal of arterial blood samples to measure blood gases.

Heart rate and systolic, diastolic and mean arterial blood pressures as well as mean and pulsatile carotid artery blood flows were continuously monitored on a polygraph (CRW, Erasmus University, Rotterdam, The Netherlands). Vascular conductances were calculated by dividing respective blood flows (ml min^{-1}) by mean arterial blood pressure (mmHg), multiplied by one hundred and expressed as 10^{-2} ml min^{-1} mmHg^{-1}. During the experiment, body temperature was maintained at $37\pm1°C$ by a heating pad and the animal was infused with physiological saline to compensate for fluid losses.

2.2.2   Distribution of carotid blood flow

The distribution of common carotid blood flow into tissue (capillary) and arteriovenous anastomotic fractions was determined in 13 pigs (later receiving vehicle, n=7 or BIBN4096BS, n=6) with radioactive microspheres (diameter: 15.5 ± 0.1 µm; S.D.), labelled with ^{141}Ce, ^{113}Sn, ^{103}Ru, ^{95}Nb or ^{46}Sc (NEN Dupont, Boston, USA). For each measurement, a suspension of about 200,000 microspheres, labelled with one of the isotopes, was mixed and injected into the carotid artery. At the end of the experiment, the animal was killed using an overdose of pentobarbital and the heart, kidneys, lungs and different cranial tissues were dissected out, weighed and put in vials. The radioactivity in these vials was counted for 5 min in a γ-scintillation counter (Packard, Minaxi autogamma 5000), using suitable windows for discriminating the different isotopes (^{141}Ce: 120-167 KeV, ^{113}Sn: 355-435 KeV, ^{103}Ru: 450-548 KeV, ^{95}Nb: 706-829 KeV and ^{46}Sc: 830-965 KeV). All data were processed by a set of specially designed computer programs (Saxena *et al.*, 1980).

The distribution of total carotid blood flow to different tissues (Q_{tis}) was calculated by the formula: $Q_{tis} = (I_{tis}/I_{total}) \times Q_{carotid}$, where I_{tis} is tissue radioactivity, I_{total} is the sum of radioactivity counted in tissues and $Q_{carotid}$ is the total common carotid blood flow at the time of microsphere injection. Since little or no radioactivity was detected in the heart or kidneys, it can be assumed that all microspheres trapped

in lungs reach the lungs from the venous side after escaping via carotid arteriovenous anastomoses. Therefore, the amount of radioactivity in the lungs can be used as an index of the arteriovenous anastomotic fraction of carotid blood flow (Saxena *et al.*, 1980; 1982).

2.2.3   Determination of plasma concentration of CGRP

Jugular venous blood samples were obtained from 12 pigs, receiving vehicle or BIBN4096BS (n=6 each). Four of these animals (2 each for vehicle and BIBN4096BS) had been used for carotid haemodynamic experiments, while the other 8 were separate experiments using the same protocol except that the radioactive microspheres were not used. Blood was transferred immediately into a polypropylene tube containing ethylene dinitro-tetraacetic acid (1 mg ml^{-1} of blood) and aprotinin (500 KIU ml^{-1} of blood). Aprotinin was used to inhibit endogenous plasma proteases, since we observed that CGRP is not detectable in biological samples without aprotinin (unpublished). After centrifugation at 1600 g for 15 min, plasma samples were coded and stored at -80°C until CGRP measurements were performed. The person measuring CGRP concentrations remained blind to the treatments, until all data had been collated.

CGRP was extracted from plasma using a C_{18} SEP-COLUMN, dried by lypholisation, and measured by radioimmunoassay (Dwenger, 1984), as per protocol of the Peninsula Laboratories, Inc (Belmont, CA, U.S.A.). The recovery of CGRP from the extraction procedure was ascertained by assaying control samples paired with a duplicate sample spiked with known quantities of CGRP. The column recovery values were 85, 79, 81, 89 and 92% (Mean=85.2; Standard deviation=5.4; Coefficient of variation=6.3%). The CGRP concentrations measured in the actual samples were, however, not corrected for the loss in the extraction procedure.

2.2.4   Experimental protocol

Following surgery and after haemodynamic condition of the animals (n=22) had been stable for 15-20 min (heart rate: 107±4 beats min^{-1}, mean arterial blood pressure: 95±2 mmHg, mean carotid blood flow: 120±12 ml min^{-1} and A-V SO$_2$ difference: 7.6±1.1%), phenylephrine was infused into the right common carotid artery at a rate of 10 µg kg^{-1} min^{-1} for 10 min, followed by 3-6 µg kg^{-1} min^{-1} throughout the rest of the experiment. The latter dose of phenylephrine was chosen so that the external

jugular venous oxygen saturation was between 60-70% and mean carotid blood flow was about 40% of the original value. After a period during which haemodynamic variables remained constant for at least 60 min (heart rate: 130 ± 4 beats min^{-1}, mean arterial blood pressure: 105 ± 2 mmHg, mean carotid blood flow: 48 ± 5 ml min^{-1} and A-V SO$_2$ difference: $31\pm2.3\%$; n=22), the animals received consecutive infusions (0.15, 0.45, 1.5 and 4.5 ml, i.c. during 3 min each) of capsaicin vehicle (see Compounds and kits section). It is important to mention that the vehicle of capsaicin was devoid of any systemic and carotid haemodynamic responses (data not shown).

Five to 10 min after the last infusion of capsaicin vehicle, blood samples were obtained for the measurements of blood gases and CGRP concentration and values of heart rate, arterial blood pressure and total carotid blood flow and conductance were collated (baseline values; 11 pigs each for vehicle and BIBN4096BS). In 12 of the 22 pigs (6 each for vehicle and BIBN4096BS) the first batch of radioactive microspheres was injected for determining the baseline distribution of carotid blood flow. The animals then received consecutive infusions of capsaicin (0.3, 1, 3 and 10 µg kg^{-1} min^{-1}, i.c. for 3 min each) and heart rate, arterial blood pressure and total carotid blood flow were determined at the end of each infusion. In addition, after the last infusion of capsaicin (10 µg kg^{-1} min^{-1}), blood gases, plasma CGRP concentration and carotid blood flow distribution were measured as described above (control values). Subsequently, a recovery period of 20 min was allowed until all haemodynamic parameters had returned to baseline levels. At this point, the animals were divided into two groups, which were treated with i.v. infusions (rate: 0.5 ml min^{-1} for 10 min) of either vehicle (three times 5 ml of acidified distilled water) or BIBN4096BS (100, 300 and 1000 µg kg^{-1}). Ten min after each infusion, capsaicin was given and haemodynamic and biochemical variables were measured again, as described above.

2.2.5   Data presentation and statistical analysis

All data are presented as mean±s.e.mean, unless stated otherwise. The statistical analysis was performed using the SPSS package for windows (version 10.0; SPSS Inc., Chicago, IL, USA). The significance of changes within one group (vehicle or BIBN4096BS) was analysed with repeated-measures ANOVA, followed by Greenhouse-Geisser correction for serial autocorrelation (Ludbrook, 1994) and

Bonferroni correction for multiple comparisons (Overall & Doyle, 1996). The significance of the between-group changes (vehicle versus BIBN4096BS treatments) was first analysed with repeated-measures ANOVA, including baseline measurements as a covariate (Overall & Doyle, 1994) and the Greenhouse-Geisser correction. If the two groups differed significantly, pairwise comparisons of corresponding values in the vehicle- and BIBN4096BS-treated groups were performed using univariate analysis (Overall & Atlas, 1999), followed by Bonferroni correction. Statistical significance was accepted at P<0.05 (two-tailed).

2.2.6   Ethical approval

The Ethics Committee of the Erasmus MC, Rotterdam, dealing with the use of animals in scientific experiments, approved the protocols for this investigation.

2.2.7   Compounds and kits

The following compounds were used: aprotinin (5850 KIU mg^{-1}; Roth, Karlsruhe, Germany), azaperone (Stresnil$^{®}$; Janssen Pharmaceuticals, Beerse, Belgium), BIBN4096BS (gift from Boehringer Ingelheim Pharma KG, Biberach, Germany), capsaicin, tween 80, ethanol and phenylephrine hydrochloride (all from Sigma-Aldrich Chemie b.v., Zwijndrecht, The Netherlands), ethylene dinitro-tetraacetic acid (Merck, Darmstadt, Germany), heparin sodium (to prevent blood clotting in catheters; Leo Pharmaceutical Products, Weesp, The Netherlands), midazolam hydrochloride (Dormicum$^{®}$; Hoffmann La Roche b.v., Mijdrecht, The Netherlands), phenylephrine hydrochloride (Sigma-Aldrich Chemie b.v., Zwijndrecht, The Netherlands) and sodium pentobarbital (Sanofi Sante b.v., Maasluis, The Netherlands). The radioimmunoassay kit for CGRP was purchased from Peninsula Laboratories, Inc. (Belmont, CA, U.S.A.).

Capsaicin was initially dissolved in tween 80, ethanol and physiological saline in the ratio of 0.5:1:8.5 ml, respectively. Phenylephrine was dissolved in distilled water, while BIBN4096BS was initially dissolved in 0.5 ml of 1N HCl, then diluted with 4 ml of distilled water and adjusted to pH 6.5 by 1N NaOH.

2.3   Results

2.3.1   Baseline values

Baseline values in the 22 pigs used were: heart rate, 126 ± 3 beats min^{-1}; mean arterial blood pressure, 105 ± 3 mmHg; total carotid blood flow, 40 ± 5 ml min^{-1}; total carotid vascular conductance, 39 ± 5 10^{-2} ml min^{-1} mmHg^{-1} and A-V SO$_2$ difference, $38\pm2\%$. No significant differences in baseline values were found between the two groups of animals (n=11 each) that later received vehicle or BIBN4096BS.

2.3.2   Effect of different doses of capsaicin on heart rate, blood pressure and carotid blood flow

Figure 2.1 depicts heart rate, mean arterial blood pressure and total carotid blood flow and conductance changes produced by the infusions of capsaicin (0.3, 1, 3 and 10 µg kg^{-1} min^{-1}, i.c.) before (control response) and after treatments with BIBN4096BS (100, 300 and 1000 µg kg^{-1} min^{-1}, i.v.) or the corresponding volumes of vehicle. In both groups of animals, capsaicin elicited dose-dependent increases in mean arterial blood pressure as well as total carotid blood flow and conductance, without significantly affecting heart rate. These effects of capsaicin remained essentially unchanged after the administration of vehicle (0.5 ml), except that a slight attenuation was noticed in the increases in carotid blood flow and conductance after the third dose of vehicle. In contrast, BIBN4096BS produced a dose-dependent attenuation of capsaicin-induced increases in total carotid blood flow and conductance, but not in blood pressure (Figure 2.1).
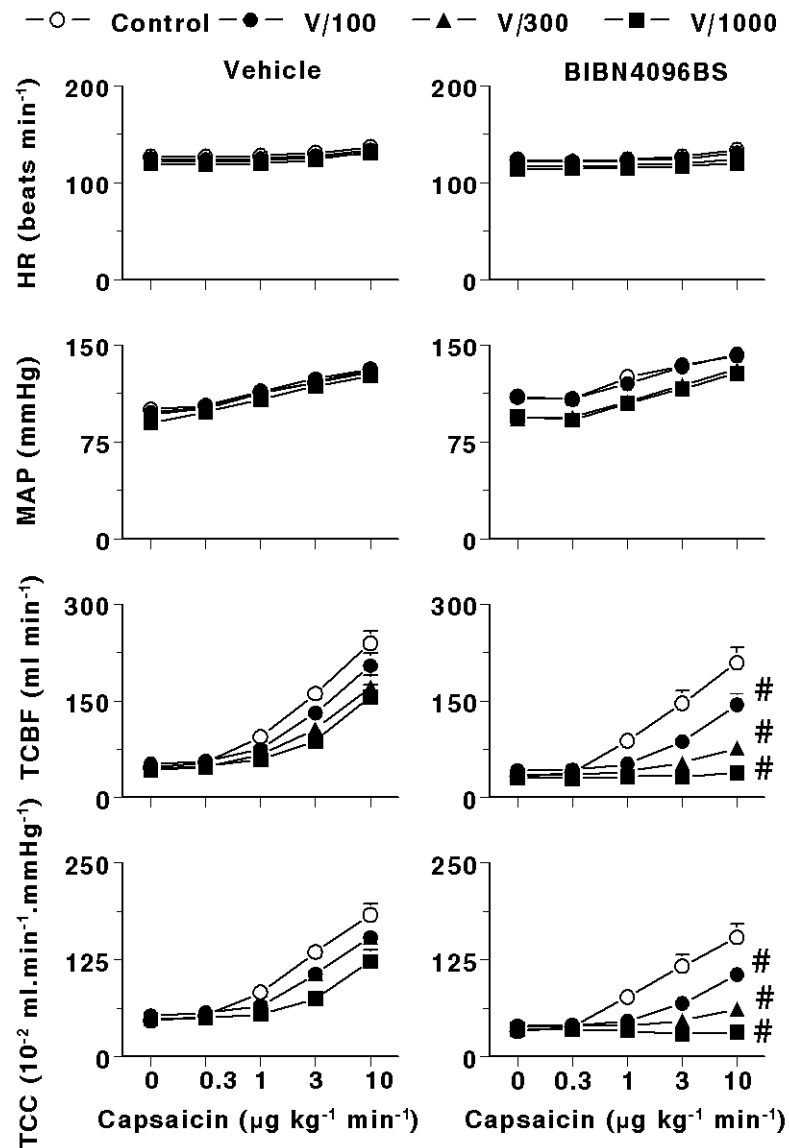
Figure 2.1. Heart rate (HR), mean arterial blood pressure (MAP) and total carotid blood flow (TCBF) and vascular conductance (TCC) values at baseline (B) and following infusions of capsaicin (0.3, 1, 3, 10 μg kg^{-1} min^{-1}, i.c.) in anaesthetised pigs before (Control) and after i.v. administrations of vehicle (V, 5 ml three times; n=11) or BIBN4096BS (100, 300 and 1000 μg kg^{-1}, n=11). All values are expressed as mean$\pm$s.e.mean. #, P<0.05 vs. response after the corresponding volume of vehicle.

2.3.3   Carotid haemodynamic changes following capsaicin infusion

The carotid haemodynamic effects observed after the highest infusion ($10\ \mu g\ kg^{-1}\ min^{-1}$) of capsaicin were examined in more detail in animals receiving vehicle or BIBN4096BS.

2.3.4   Effect on carotid blood flow and pulsations

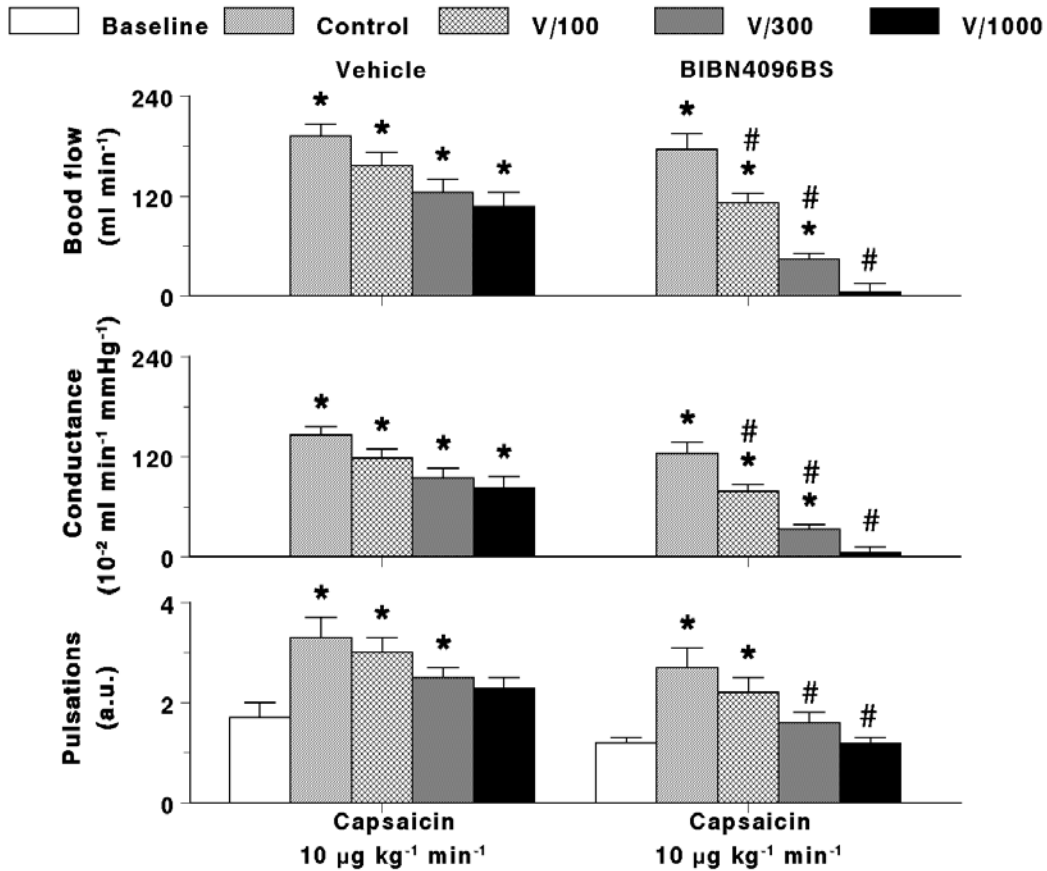As shown in Figure 2.2, i.c. infusions of capsaicin ($10\ \mu g\ kg^{-1}\ min^{-1}$) clearly increased carotid blood flow and conductance (both depicted as maximum absolute changes) as well as pulsations. In animals treated with vehicle, there was some decrease in the responses to capsaicin, but these responses were significantly more attenuated in animals treated with BIBN4096BS, particularly the two highest doses.

2.3.4   Fractionation of carotid blood flow and vascular conductance

In both vehicle and BIBN4096BS groups, capsaicin ($10\ \mu g\ kg^{-1}\ min^{-1}$, i.c.) significantly increased total carotid blood flow and conductance as well as those distributed to arteriovenous anastomoses and capillaries. The increases from baseline values in blood flows and vascular conductances were, respectively: total carotid, 494±59% and 362±40%; arteriovenous anastomotic fraction, 726±282% and 505±188% and capillary fraction, 526±48% and 389±32% (n=13 in each case).

The effects of BIBN4096 as well as of its vehicle on the carotid haemodynamic responses to capsaicin are illustrated in Figure 2.3. Compared to the corresponding volumes of vehicle, the increases in total, arteriovenous anastomotic as well as capillary blood flows and vascular conductances were clearly antagonised after the two highest infusions (300 and $1000\ \mu g\ kg^{-1}\ min^{-1}$) of BIBN4096BS.

Figure 2.4 shows that capsaicin ($10\ \mu g\ kg^{-1}\ min^{-1}$, i.c.) increased vascular conductances to the different cranial tissues, including the skin, ear, skeletal muscles, fat, bone, eye, tongue and dura mater, but not in the brain or salivary glands. As has been described with 5-hydroxytryptamine (Saxena & Verdouw, 1982), the increase in skin blood flow was most likely responsible for the redness of skin on the side of capsaicin infusion (not shown in the Figure). These effects of capsaicin were significantly and dose-dependently antagonised by BIBN4096BS (100, 300 and $1000\ \mu g\ kg^{-1}\ min^{-1}$, i.v.), but not by the corresponding volumes of vehicle.

Figure 2.2. Maximum changes in carotid blood flow, vascular conductance and pulsations measured at baseline and following infusions of capsaicin (10 μg kg^{-1} min^{-1}, i.c.) given in anaesthetised pigs before (Control) and after i.v. administrations of vehicle (V, 5 ml three times; n=11) or BIBN4096BS (100, 300 and 1000 μg kg^{-1}, n=11). All values are expressed as mean±s.e.mean. a.u., Arbitrary units. *, P < 0.05 vs. baseline values; #, P<0.05 vs. response after the corresponding volume of vehicle.
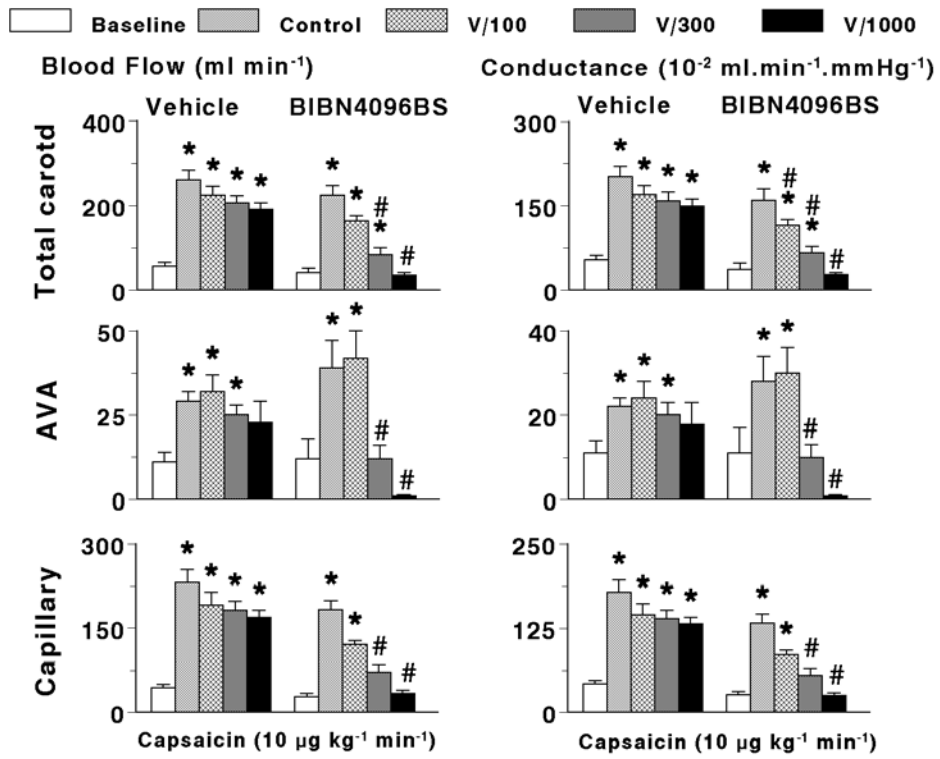
Figure 2.3. Total carotid, arteriovenous anastomotic (AVA) and capillary blood flows (*left panel*) and vascular conductances (*right panel*) measured at baseline and following infusions of capsaicin (10 μg kg^{-1} min^{-1}, i.c.) given in anaesthetised pigs before (Control) and after i.v. administrations of vehicle (V, 5 ml three times; n=7) or BIBN4096BS (100, 300 and 1000 μg kg^{-1}, n=6). All values are expressed as mean±s.e.mean. *, $P < 0.05$ vs. baseline values; #, $P < 0.05$ vs. response after the corresponding volume of vehicle.
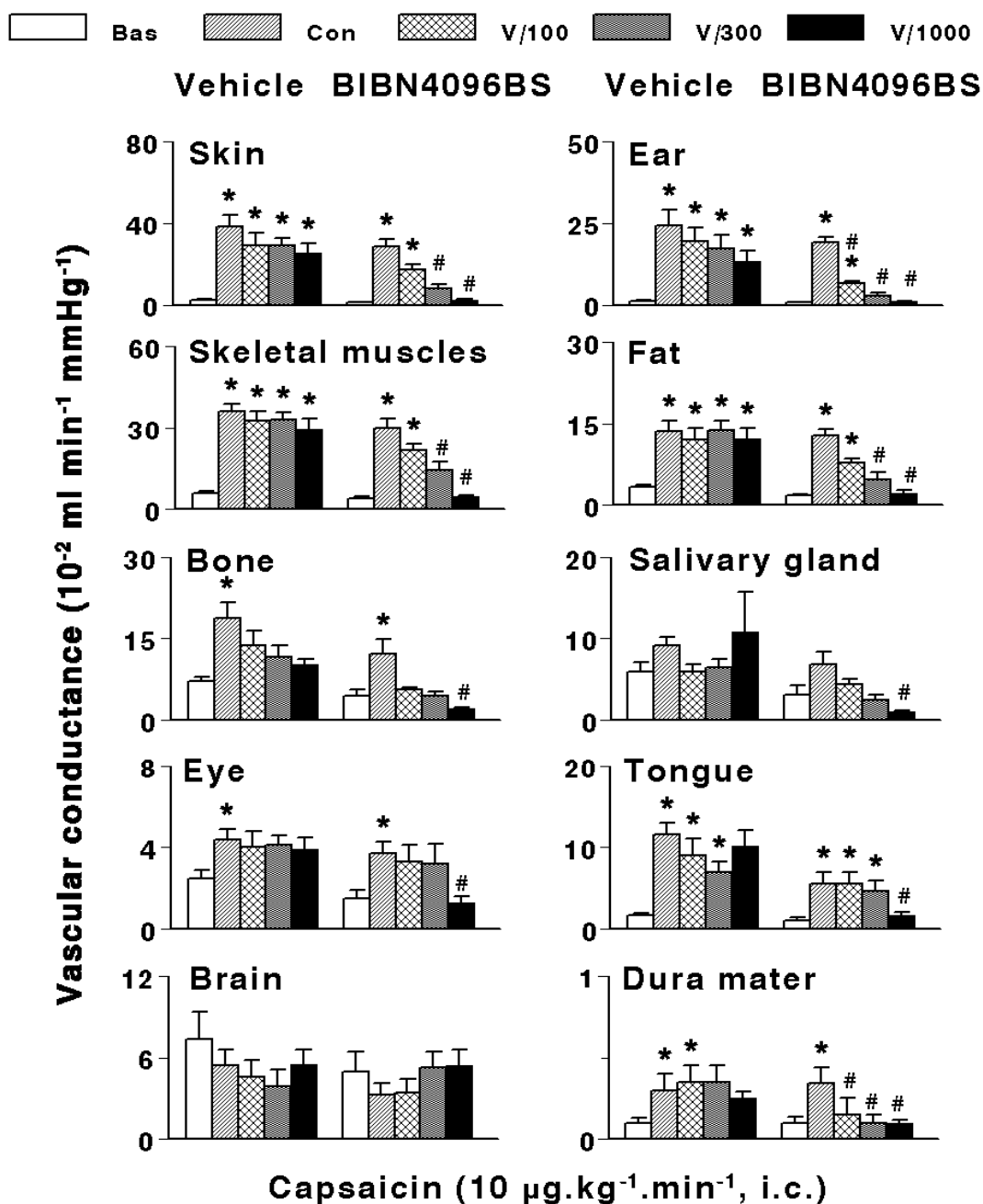
Figure 2.4. Distribution of carotid vascular conductances to head tissues measured at baseline (Bas) and following infusions of capsaicin (10 μg kg^{-1} min^{-1}, i.c.) given in anaesthetised pigs before (Con) and after i.v. administrations of vehicle (V, 5 ml three times; n=7) or BIBN4096BS (100, 300 and 1000 μg kg^{-1}, n=6). All values are expressed as mean±s.e.mean.   *, P < 0.05 vs. baseline values; #, P<0.05 vs. response after the corresponding volume of vehicle.

2.3.5   Difference between arterial and jugular venous oxygen saturations (A-V SO$_2$ difference)

Consistent with the increase in arteriovenous anastomotic blood flow, capsaicin (10 µg kg^{-1} min^{-1}, i.c.) significantly decreased A-V SO$_2$ difference from baseline values of 38±2% to 4.5±0.4% (n=22). This response remained unaffected in animals treated with vehicle, but was dose-dependently antagonised by BIBN4096BS (Figure 2.5).

Figure 2.5. Differences between arterial and jugular venous oxygen saturations (A-V SO$_2$ difference) measured at baseline and following infusions of capsaicin (10 µg kg^{-1} min^{-1}, i.c.) given in anaesthetised pigs before (Control) and after i.v. administrations of vehicle (V, 5 ml three times; n=11) or BIBN4096BS (100, 300 and 1000 µg kg^{-1}, n=11). All values are expressed as mean±s.e.mean. *, P < 0.05 vs. baseline values;   #, P<0.05 vs. response after the corresponding volume of vehicle.
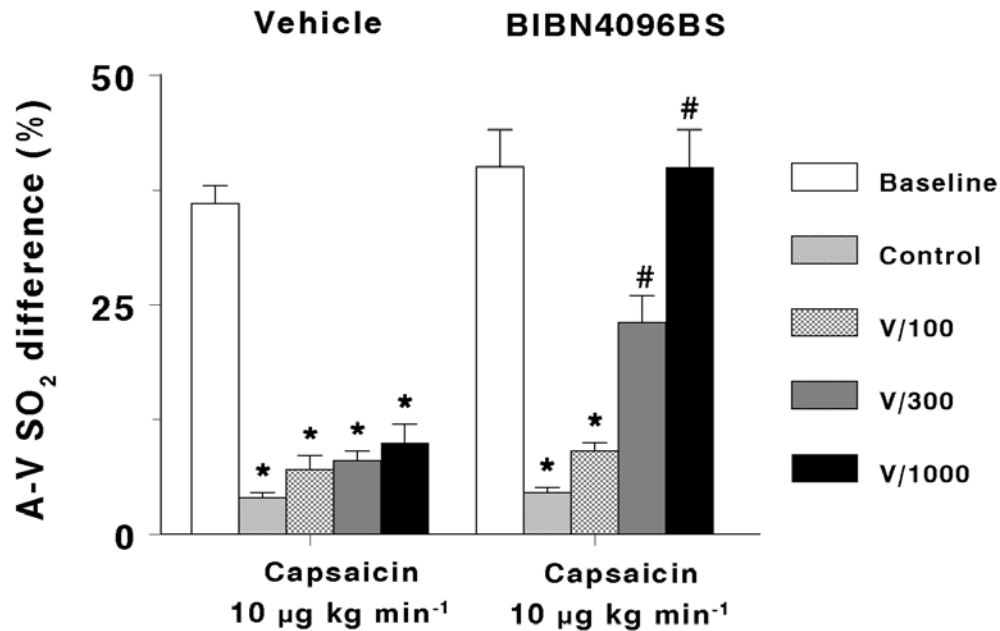
2.3.6   Jugular venous plasma concentrations of CGRP

In the 12 pigs used for this purpose, the baseline value of CGRP concentration in jugular venous plasma was 27±2 pg ml^{-1} and following capsaicin infusion (10 µg kg^{-1} min^{-1}, i.c.) it increased by 119±17% to 58±5 pg ml^{-1}. Figure 2.6 shows the effects of capsaicin (10 µg kg^{-1} min^{-1}, i.c.) on jugular venous plasma concentration of CGRP in pigs receiving either three i.v. infusions of vehicle (5 ml each) or

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BIBN4096BS (100, 300 and 1000 μg kg^{-1}).   Capsaicin increased plasma CGRP
concentration in both animal groups by a similar magnitude and this increase was not
attenuated in either vehicle- or BIBN4096BS-treated group of animals.   Interestingly,
following the two highest doses of BIBN4096BS (300 and 1000 μg kg^{-1}, i.v.) there
was even a potentiation of capsaicin-induced increases in plasma CGRP
concentrations (control response: 138±29%; response after BIBN4096BS: 211±30%
and 211±38%, respectively; n=6).
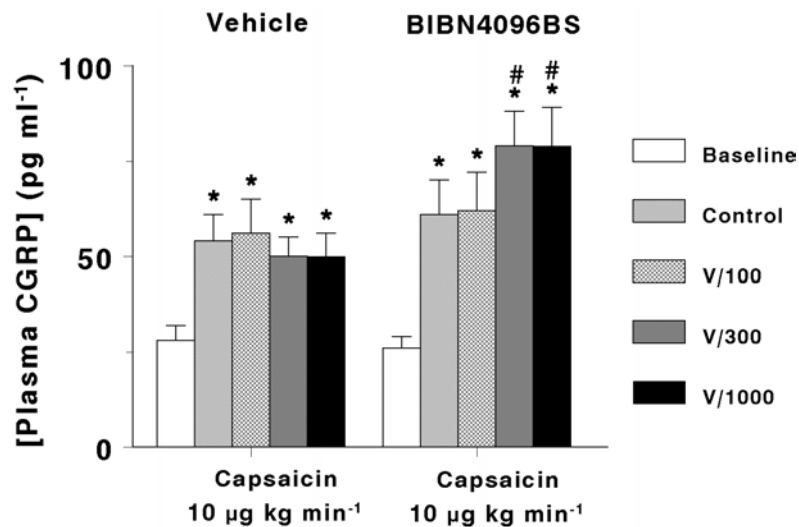
Figure 2.6. Jugular venous plasma CGRP concentrations
measured at baseline and after infusions of capsaicin (10
μg kg^{-1} min^{-1}, i.c.) given in anaesthetised pigs before (Control)
and after i.v. administrations of vehicle (V, 5 ml three times;
n=7) or BIBN4096BS (100, 300 and 1000 μg kg^{-1}, n=6).
All values are expressed as mean±s.e.mean.   *, P < 0.05 vs.
baseline values;   #, P<0.05 vs. response after the
corresponding volume of vehicle.

2.4 Discussion

2.4.1 General

Although there is much debate about the pathogenesis of migraine, there seems to be a general agreement regarding its neurovascular nature (Goadsby & Edvinsson, 1993; De Vries *et al.*, 1999a; Goadsby *et al.*, 2002b; Villalón *et al.*, 2002). Thus, there is a release of vasoactive peptides producing intense cranial vasodilatation, increased arterial pulsations and a sterile inflammatory reaction with pain (Moskowitz *et al.*, 1989; De Vries *et al.*, 1999a). Amongst these neuropeptides, CGRP is considered as a biological marker in migraine pathogenesis (van Rossum *et al.*, 1997; Goadsby *et al.*, 2002b; Hagner *et al.*, 2002c). Moreover, stimulation of trigeminal sensory neurones with electrical procedures or chemical substances, like capsaicin, releases endogenously-stored CGRP (Buzzi *et al.*, 1995; Eltorp *et al.*, 2000) that, in turn, dilates cranial vessels (Williamson & Hargreaves, 2001), including carotid arteriovenous anastomoses (Van Gelderen *et al.*, 1995). In addition, CGRP may also facilitate sensory nerve transmission between the first and second order afferent input from these vessels during migraine headache (Gulbenkian *et al.*, 2001; Goadsby *et al.*, 2002b; Smith *et al.*, 2002). On this basis, it is reasonable to assume that CGRP receptor antagonists can be a novel approach to antimigraine therapy. In this respect, recent *in vitro* studies have shown that, BIBN4096BS, a potent and 'silent' CGRP receptor antagonist (Doods *et al.*, 2000), inhibits CGRP-induced dilatation of isolated cranial blood vessels (Edvinsson *et al.*, 2002; Verheggen *et al.*, 2002). BIBN4096BS can also effectively antagonise CGRP-induced carotid vasodilatation in anaesthetised pigs (Kapoor *et al.*, 2003b). Therefore, it seems important to investigate the effects of BIBN4096BS on the carotid haemodynamic responses produced by endogenous CGRP released by capsaicin in a porcine model predictive of antimigraine activity (Saxena, 1995; De Vries *et al.*, 1999a; Tfelt-Hansen *et al.*, 2000). Our results show that: (i) i.c. administration of capsaicin increased blood pressure, but dilated carotid arteriovenous anastomoses and arterioles, together with an increase in carotid pulsations and a narrowing of A-V SO_2 difference as well as an elevation of jugular venous plasma CGRP concentration; and (ii) BIBN4096BS dose-dependently antagonised the changes in carotid haemodynamics and A-VSO_2 difference caused by

capsaicin, but it enhanced the capsaicin-induced increase in jugular venous plasma CGRP concentration.

2.4.2 Systemic haemodynamic responses to capsaicin

The widespread distribution of CGRP immunoreactivity in cardiovascular tissues suggests that CGRP may play a role in the regulation of systemic and regional haemodynamics (Bell & McDermott, 1996; Hagner *et al.*, 2002c). In fact, several *in vivo* studies have evidenced a hypotensive response to CGRP due to its potent vasodilator action (Bell & McDermott, 1996; Shen *et al.*, 2001). In contrast, our study shows a significant increase in mean blood pressure following i.c. capsaicin, and this increase was not abolished by BIBN4096BS. Despite the absence of clear tachycardic responses to i.c. capsaicin, the simplest interpretation of these findings may be that the vasopressor response to capsaicin is not mediated via CGRP receptors, but is rather due to an interaction with vasoconstrictor mechanisms. Indeed, not only do high subcutaneous doses (50 mg kg^{-1}) of capsaicin increase plasma CGRP concentrations, but also plasma catecholamines, neurokinin A and neuropeptide Y concentrations (Alving *et al.*, 1991).

2.4.3 Carotid haemodynamics

Stimulation of the trigeminal ganglion increases cerebral blood flow and releases endogenous vasoactive neuropeptides, including CGRP (Goadsby *et al.*, 1988). Vasoactive neuropeptides are also released from sensory afferent nerves by capsaicin, but its relaxant effect on isolated cerebral blood vessels is mediated by CGRP, rather than by substance P or neurokinin A (Jansen *et al.*, 1990; O'Shaughnessy *et al.*, 1993; Jansen-Olesen *et al.*, 1996). These findings are in full agreement with our results in anaesthetised pigs showing dose-dependent vasodilator responses to capsaicin in the carotid circulation, including arteriovenous anastomoses and arterioles. Admittedly, as reported earlier (Szallasi & Blumberg, 1999), vasodilator responses to capsaicin tended to wear off in vehicle-treated animals, suggestive of tachyphylaxis. This tachyphylaxis was rather limited, possibly due to a neuronal reuptake of released CGRP into capsaicin-sensitive perivascular nerves (Sams-Nielsen *et al.*, 2001). However, compared to the vehicle-treated animals, the carotid haemodynamic effects of capsaicin were clearly much more attenuated by the potent and selective CGRP receptor antagonist BIBN4096BS (Doods *et al.*, 2000; Wu *et al.*, 2000; Doods, 2001;

Wu *et al.*, 2002). BIBN4096BS has also been demonstrated to effectively block the relaxation of blood vessels by CGRP, both *in vitro* (Doods *et al.*, 2000; Edvinsson, 2002; Moreno *et al.*, 2002; Verheggen *et al.*, 2002; Wu *et al.*, 2002) and *in vivo* (Doods *et al.*, 2000), including the porcine carotid vascular bed (Kapoor *et al.*, 2003a). Therefore, it is clear that carotid vasodilatation by capsaicin in the present investigation is mediated by the release of CGRP.

2.4.4   A-V SO$_2$ difference

During the headache phase of migraine, the A-V SO$_2$ difference is abnormally low, presumably due to an opening of arteriovenous shunts (Heyck, 1969). Thus, a reduction of carotid arteriovenous anastomotic blood flow, with a consequent normalisation of the A-V SO$_2$ difference, makes our porcine vascular model highly predictive of antimigraine activity (Saxena, 1987; Saxena, 1995; De Vries *et al.*, 1999a). In the present study, i.c. infusions of capsaicin significantly decreased A-V SO$_2$ difference together with dilatation of carotid arteriovenous anastomoses. Since both these effects of capsaicin were effectively blocked by BIBN4096BS, it confirms that capsaicin-induced responses are mediated via the release of CGRP. Indeed, CGRP also decreases A-V SO$_2$ difference and this effect is antagonised by BIBN4096BS (Kapoor *et al.*, 2003b).

2.4.5   Plasma concentrations of CGRP

The release of CGRP by capsaicin is mediated by selective activation of the Aδ- and C-fibre sensory neurones via vanilloid receptors (Caterina *et al.*, 1997; Ebersberger *et al.*, 1999; Eltorp *et al.*, 2000). Our results showing an increase in plasma concentrations of CGRP after capsaicin (see Figure 2.6) are consistent with the above observations. Interestingly, not only did BIBN4096BS fail to block capsaicin-induced CGRP release, but also there was a modest enhancement of CGRP release. There is evidence for uptake of CGRP into perivascular, capsaicin-sensitive neurones in the guinea pig isolated basilar artery (Sams-Nielsen *et al.*, 2001). Therefore, it may well be that blockade of prejunctional 'inhibitory' CGRP autoreceptors by BIBN4096BS led to increased release of CGRP by capsaicin, similar to the modulation of sympathetic neurotransmission by presynaptic α-adrenoceptors (Langer, 1980).

        It may be noted that plasma CGRP concentrations measured by us at baseline (27±2 pmol ml^{-1}, n=12) as well as after capsaicin treatment (58±5 pmol ml^{-1}, n=12)

are in agreement with those previously reported in pigs (Table 2.1) (Alving *et al.*, 1991; Arden *et al.*, 1994; Kallner *et al.*, 1998).

Table 2.1. Plasma CGRP concentration range (pg ml^{-1}) in pigs

Baseline	Capsaicin	Sampled from	Reference
10	36	Femoral artery	Alving et al. (Alving *et al.*, 1991)
11-16	Not measured	Femoral artery and interventricular vein	Kallner *et al.* (Kallner *et al.*, 1998)
4-12	Not measured	Carotid artery	Arden et al. (Arden *et al.*, 1994)
14-38	27-88	External jugular vein	Present investigation

2.4.6   Possible clinical implications

Lastly, we would like to consider the possible clinical implications of our results with BIBN4096BS within the context of antimigraine therapy. Indeed, the trigeminovascular system, a functional network of cranial blood vessels and their trigeminal innervation, seems to be activated during migraine (Goadsby *et al.*, 2002b), thereby provoking CGRP release and cranial blood vessel dilatation. Thus, a blockade of the release and/or the effects of CGRP are likely to provide novel avenues for developing antimigraine drugs without associated vasoconstriction. BIBN4096BS may be such a compound and the present findings demonstrating that it effectively antagonises the carotid vasodilator responses elicited by capsaicin are indeed encouraging. Obviously, the results of currently undergoing clinical trials with BIBN4096BS are awaited with great interest; these would be crucial in determining not only the role of CGRP in the pathophysiology of migraine, but also of such compounds as therapeutic agents.

CHAPTER 3

Effects of BIBN4096BS on cardiac output distribution and on CGRP-induced carotid haemodynamic responses in the pig

3   Effects of BIBN4096BS on cardiac output distribution and on CGRP-induced carotid haemodynamic responses in the pig

Abstract: Calcitonin gene related peptide (CGRP) seems to be involved in the pathogenesis of migraine, since plasma CGRP levels increase during the headache phase. In the present study, we investigated the effects of a novel CGRP receptor antagonist, BIBN4096BS (1-piperidinecarboxamide, N-[2-[[5-amino-1-[[4-(4-pyridinyl)-1-pipera-zinyl]carbonyl] pentyl] amino]-1-[(3,5-dibromo-4-hydroxyphenyl) methyl]- 2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2H)-quinazolinyl) (Kapoor *et al.*, 2003b), on the regional cardiac output distribution and on the carotid haemodynamic changes induced by α-CGRP in anaesthetised pigs. Treatment with BIBN4096BS (100, 300 and 1000 $\mu g.kg^{-1}$, i.v.) did not affect heart rate, mean arterial blood pressure or systemic vascular conductance, but a small decrease in cardiac output was noticed; the latter was, however, not significantly different from that in vehicle-treated animals. The highest dose of BIBN4096BS moderately decreased vascular conductance in the lungs, kidneys, spleen and adrenals. Vascular conductance in other tissues, including the brain, heart, gastrointestinal system, skin and skeletal muscles remained unchanged. Intracarotid artery infusions of α-CGRP (10, 30 and 100 $pmol.kg^{-1}.min^{-1}$ during 3 min) increased total carotid blood flow and conductance, but decreased arterial blood pressure. These responses were dose-dependently blocked by BIBN4096BS. The above results show that BIBN4096BS is a CGRP receptor antagonist in the porcine carotid and systemic circulations, but the endogenous CGRP does not seem to play an important physiological role in regulating basal vascular tone. These findings suggest that BIBN4096BS may have therapeutic usefulness in migraine.

3.1   Introduction

Calcitonin gene related peptide (CGRP), a 37 amino acid neuropeptide generated by alternative splicing of the calcitonin gene (Amara *et al.*, 1982), is widely distributed in the body, including in trigeminal sensory nerve fibres innervating central and peripheral blood vessels, where it is co-localised with other vasoactive neuropeptides, such as substance P and neurokinin A (Gulbenkian *et al.*, 1995; Gulbenkian *et al.*, 2001). CGRP is a potent vasodilator agent in a wide variety of tissues (Brain & Williams, 1985; van Rossum *et al.*, 1997; Juaneda *et al.*, 2000; Poyner & Marshall, 2001) and, although exogenous α-CGRP has potent systemic and regional haemodynamic effects (Gardiner *et al.*, 1990), the physiological role of endogenous CGRP is not clear (Shen *et al.*, 2001). This is mainly due to the unavailability of potent and selective CGRP receptor antagonists; the most widely used CGRP receptor

antagonist thus far, CGRP-(8-37) is not very potent and displays partial agonist properties (Wisskirchen *et al.*, 1998; Waugh *et al.*, 1999). Clearly, the advent of 'silent', selective and potent non-peptide CGRP receptor antagonists would be valuable in this regard.

Interestingly, CGRP has been implicated in the pathogenesis of migraine (Goadsby *et al.*, 1990; Ashina *et al.*, 2000; Edvinsson, 2001b; Durham & Russo, 2002), and it can mediate neurogenic dilatation of cranial blood vessels as well as sensory nerve transmission between the first and second order afferent input from these vessels during migraine headache (Gulbenkian *et al.*, 2001; Williamson & Hargreaves, 2001; Goadsby *et al.*, 2002b; Smith *et al.*, 2002). Significantly, plasma levels of CGRP, but not of other neurotransmitter (e.g., neuropeptide Y, vasoactive intestinal peptide or substance P), are elevated during migraine and, after sumatriptan, these levels are normalised paralleling the resolution of headache (Goadsby *et al.*, 1990; Goadsby & Hoskin, 1999). Therefore, inhibition of α-CGRP release or blockade of α-CGRP-induced vasodilatation may be a novel approach in the management of acute migraine headache.

Doods and colleagues (2000) have recently described a small molecule CGRP receptor antagonist, BIBN4096BS (1-piperidinecarboxamide, N-[2-[[5-amino-1-[[4-(4-pyridinyl)-1-piperazinyl]carbonyl]pentyl] amino]- 1-[(3,5-dibromo-4-hydroxyphenyl) methyl]- 2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2H)-quinazolinyl) (Kapoor *et al.*, 2003b), which possesses over 200 fold higher affinity for human (SK-N-MC cells; K_i: 14 pM) than for rat (spleen; K_i: 3.4 nM) CGRP receptors. BIBN4096BS as well as the endogenous ligand CGRP and its analogues concentration-dependently displace (Kawasaki *et al.*, 1990; Bard *et al.*, 1993; Goadsby & Knight, 1997; Yu *et al.*, 1997; Bhalla *et al.*, 2002)BIBN4096BS from SK-N-MC cell membranes with the rank order of affinity: BIBN4096BS > human α-CGRP = human β-CGRP > [Cys(Et)^{2,7}] human α-CGRP > adrenomedullin (high affinity site) = human α-CGRP$_{8-37}$ = human β-CGRP$_{8-37}$ >> calcitonin = amylin (Schindler & Doods, 2002). The compound inhibits vasodilatation evoked by trigeminal ganglion stimulation in marmosets (Doods *et al.*, 2000) and by CGRP in several human isolated blood vessels (Edvinsson *et al.*, 2002; Moreno *et al.*, 2002; Verheggen *et al.*, 2002). The purpose of the present study in anaesthetised pigs was to investigate the effects of BIBN4096BS on: (i) the complete distribution of cardiac

output to assess the potential role of endogenous CGRP in regulating basal vascular tone and thereby the cardiovascular safety of BIBN4096BS, and (ii) the haemodynamic responses produced by intracarotid arterial (i.c.) infusion of α-CGRP in a model predictive of antimigraine activity (Saxena, 1995; De Vries *et al.*, 1999a).

3.2   Materials and methods

3.2.1   General

After an overnight fast, 25 domestic pigs (Yorkshire x Landrace, females, 10-14 kg) were sedated with intramuscular injections of azaperone (120 mg) and midazolam hydrochloride (10 mg) and then anaesthetised with sodium pentobarbital (600 mg, i.v.). After tracheal intubation, the animals were connected to a respirator (BEAR 2E, BeMeds AG, Baar, Switzerland) for intermittent positive pressure ventilation with a mixture of room air and oxygen. Respiratory rate, tidal volume and oxygen supply were adjusted to keep arterial blood gas values within physiological limits (pH: 7.35-7.48; pCO_2: 35-48 mmHg; pO_2: 100-120 mmHg). Anaesthesia was maintained with a continuous i.v. infusion of sodium pentobarbital ($12\text{-}20\ mg.kg^{-1}.h^{-1}$). Heart rate was measured with a tachograph (CRW, Erasmus University, Rotterdam, The Netherlands) triggered by electrocardiogram signals. A catheter was placed in the inferior vena cava via the right femoral vein for the administration of vehicle and BIBN4096BS. Another catheter was placed in the aortic arch via the left femoral artery for the measurement of arterial blood pressure (Combitrans disposable pressure transducer; Braun, Melsungen, Germany) and arterial blood withdrawal for the measurement of blood gases (ABL-510; Radiometer, Copenhagen, Denmark). During the experiment, body temperature was kept around 37°C and the animal was continuously infused with physiological saline to compensate for fluid losses.

Heart rate and systolic, diastolic and mean arterial blood pressure as well as the pulsatile and mean carotid artery blood flows (see later) were continuously monitored on a polygraph (CRW, Erasmus University, Rotterdam, The Netherlands).

3.2.2   Cardiac output and its distribution

Cardiac output was measured by the thermodilution method using a 6F Swan-Ganz catheter (Braun Melsungen AG, Melsungen, Germany) introduced into the pulmonary artery via the left femoral vein.

The distribution of cardiac output was determined with 15.5±0.1 (S.D.) µm diameter microspheres labelled with ^{141}Ce, ^{113}Sn, ^{103}Ru, ^{95}Nb or ^{46}Sc (NEN Dupont, Boston, USA).   For each measurement, a suspension of about 1,000,000 microspheres, labelled with one of the isotopes, was injected into the left ventricle via a catheter guided by way of the left carotid artery. Starting 15 s before microsphere injection and lasting 70 s, a reference arterial blood sample was withdrawn (Withdrawal pump, Harvard Apparatus Company, Southnatick, Mass, USA; rate: 6 ml.min^{-1}) via a catheter placed into the right femoral artery. An infusion of the corresponding volume of Haemaccel compensated blood loss during this procedure.

At the end of the experiment, the animal was killed using an overdose of pentobarbital. Subsequently, a number of tissues (lungs, kidneys, heart, stomach, small intestine, spleen, liver, adrenals, brain, skin and skeletal muscles) were dissected out, weighed and put into vials. The radioactivity in these vials was counted for 5 min in a γ-scintillation counter (Packard, Minaxi autogamma 5000), using suitable windows for the discrimination of the different isotopes (^{141}Ce: 120-167 KeV, ^{113}Sn: 355-435 KeV, ^{103}Ru: 450-548 KeV, ^{95}Nb: 706-829 KeV and ^{46}Sc: 830-965 KeV). All data were processed by a set of specially designed computer programs (Saxena *et al.*, 1980), using a personal computer. Tissue blood flows were calculated by multiplying the ratio of tissue and reference blood sample radioactivities by the blood withdrawal rate (6 ml.min^{-1}) and normalised to 100 g tissue weight. Systemic and tissue vascular conductances were calculated by dividing cardiac output (ml.min^{-1}) and tissue blood flows (ml.min^{-1}/100 g tissue), respectively, by mean arterial blood pressure (mmHg).   Radioactivity in the lungs mainly represents peripheral arteriovenous anastomotic blood flow (the non-nutrient part of the cardiac output), although a small part (1-1.5% of cardiac output) is derived from the bronchial arteries (Baile *et al.*, 1982).

3.2.3 Carotid haemodynamic responses to CGRP

Both common carotid arteries and the external jugular veins were dissected free and the accompanying vagosympathetic trunks were cut between two ligatures in order to prevent a possible influence of CGRP via baroreceptor reflexes. Pulsatile and mean blood flows were measured in the right common carotid artery with a flow probe (internal diameter: 2.5 mm) connected to a sine-wave electromagnetic flow meter (Transflow 601-system, Skalar, Delft, The Netherlands). The amplitude of carotid blood flow signals provided an index of carotid flow pulse. Carotid vascular conductance was calculated by dividing carotid blood flow (ml.min^{-1}) by mean arterial blood pressure (mmHg).

The right external jugular vein was catheterised for obtaining jugular venous blood samples to determine blood gases. Two hub-less needles, connected to polyethylene tubes, were inserted into the right common carotid artery and used for intracarotid (i.c.) infusions of phenylephrine (α_1-adrenoceptor agonist) and α-CGRP, respectively. It should be noted that under pentobarbital anaesthesia carotid arteriovenous anastomoses are dilated (Den Boer *et al.*, 1993) and, therefore, to elicit vasodilator responses to CGRP, a continuous infusion of phenylephrine was used throughout the experiment. We have previously reported that phenylephrine decreases total carotid blood flow and conductance exclusively due to constriction of carotid arteriovenous anastomoses (Willems *et al.*, 1999), resulting in an increase in the difference between arterial and jugular venous oxygen saturations (A-V SO$_2$ difference) (Saxena, 1987).

3.2.4 Experimental protocols

In the case of cardiac output distribution experiments (n=12), baseline values of heart rate, mean arterial blood pressure, cardiac output and its distribution to the various tissues (see above) were determined after a stabilisation period of at least 90 min. The animals were then divided into two groups (n=6 each) receiving three i.v. infusions (rate: 0.5 ml.min^{-1}) of either BIBN4096BS (100, 300 and 1000 µg.kg^{-1}) or its vehicle (5 ml of acidified distilled water); each dose was given over 10 min with an intervening period of 10 min before the next dose. At the end of each infusion, the above mentioned haemodynamic variables were collated again. Lastly, the final

measurements were made 40 min after the third dose of vehicle or BIBN4096BS (recovery).

In the case of the carotid artery experiments (n=13), phenylephrine (10 µg.kg^{-1}.min^{-1} for 10 min, followed by 3-6 µg.kg^{-1}.min^{-1} throughout the rest of the experiment) was infused into the right common carotid artery to maintain carotid blood flow at a constant low level. After a stabilisation period of at least 90 min, values of heart rate, arterial blood pressure, total carotid blood flow and A-V SO$_2$ difference were collated. The animal was then given three sequential i.c. infusions (rate: 0.083-1 ml.min^{-1}, depending on the weight of the animal) of CGRP (10, 30 and 100 pmol.kg^{-1}.min^{-1}) for 3 min and the above variables (except the A-V SO$_2$ difference, which was determined only after the highest dose) were collated again. After the highest dose of α-CGRP, a recovery period of 20 min was allowed to elapse when all haemodynamic parameters returned to baseline levels. At this point, the animals were divided into two groups receiving three i.v. infusions (rate: 0.5 ml.min^{-1}) of either BIBN4096BS (100, 300 and 1000 µg.kg^{-1}; n=7) or its vehicle (5 ml of acidified distilled water; n=6); each dose was given over a period of 10 min with an intervening period of about 10 min before the next dose. Ten min after each treatment, the values of mean arterial blood pressure, heart rate, total carotid blood flow and A-V SO$_2$ difference were collated. CGRP was infused as above after each treatment and data were collated again.

It may be mentioned that the vehicle of α-CGRP (distilled water) was devoid of any systemic and carotid haemodynamic responses (data not shown).

3.2.5   Data presentation and statistical analysis

All data have been expressed as mean±s.e.mean, unless stated otherwise. The significance of changes from baseline values within one group (vehicle or BIBN4096BS) was evaluated with Duncan's new multiple range test, once an analysis of variance (randomised block design) had revealed that the samples represented different populations (Saxena *et al.*, 1980; Steel & Torrie, 1980). The differences in baseline haemodynamic values and percent change (from baseline values) in haemodynamic variables by corresponding doses of the vehicle and BIBN4096BS (between group comparisons) were evaluated by Student's unpaired t-test. Student's unpaired t-test was also applied to compare the changes in the effects of CGRP

observed after different corresponding doses of the vehicle and BIBN4096BS. Statistical significance was accepted at P<0.05 (two-tailed).

3.2.6   Ethical approval

The Ethics Committee of the Erasmus MC, Rotterdam, dealing with the use of animals in scientific experiments, approved investigation protocols, which adhere to EEC guidelines.

3.2.7   Compounds

The following compounds were used: azaperone (Stresnil®; Janssen Pharmaceuticals, Beerse, Belgium), BIBN4096BS and human α-CGRP (Boehringer Ingelheim Pharma KG, Biberach, Germany), heparin sodium (to prevent blood clotting in catheters; Leo Pharmaceutical Products, Weesp, The Netherlands), midazolam hydrochloride (Dormicum®; Hoffmann La Roche b.v., Mijdrecht, The Netherlands), phenylephrine hydrochloride (Sigma-Aldrich Chemie b.v., Zwijndrecht, The Netherlands) and sodium pentobarbital (Sanofi Sante b.v., Maasluis, The Netherlands).

Phenylephrine and α-CGRP were dissolved in distilled water, while BIBN4096BS was initially dissolved in 0.5 ml of 1N HCl and subsequently diluted with 4 ml of distilled water, and then adjusted to pH 6.5 with 1N NaOH.

3.3   Results

3.3.1   Effect of BIBN4096BS on cardiac output and its distribution

BASELINE VALUES

Baseline values of heart rate, mean arterial blood pressure, cardiac output (expressed as cardiac index) and systemic vascular conductance in anaesthetised pigs (n=12) were: 108 ± 3 beats.min^{-1}, 102 ± 2 mmHg, 133 ± 4 ml.min^{-1}.kg^{-1} and 1491 ± 54 ml.min^{-1}.mmHg^{-1}, respectively. Baseline values of regional vascular conductances (ml.min^{-1}.mmHg^{-1}/100 g tissue) were: brain, 31 ± 3; heart, 104 ± 10; liver, 34 ± 8; stomach, 24 ± 2; lungs (mainly systemic arteriovenous anastomoses), 229 ± 37; adrenals, 138 ± 10; kidneys, 263 ± 14; spleen, 126 ± 15; skeletal muscles, 3.3 ± 0.3; and skin, 11 ± 2.

SYSTEMIC AND REGIONAL HAEMODYNAMIC CHANGES

Systemic haemodynamic values collated at baseline, after vehicle or BIBN4096BS (100, 300 and 1000 μg.kg^{-1}, i.v.) and after a 40-min recovery period are shown in Figure 3.1. There were no statistically significant differences (P>0.05) in baseline values in the vehicle and BIBN4096BS groups. Except for small decreases in heart rate by the vehicle (maximum change: 4±1%) and cardiac index by BIBN4096BS (maximum change: 19±8%), no other changes were observed. The changes in cardiac index by BIBN4096BS did not differ significantly (P>0.05) from those in the vehicle-treated animals (maximum change: 7±3%).

Figure 3.2 presents regional vascular conductances in a number of tissues in animals treated with either vehicle or BIBN4096BS (100, 300 and 1000 μg.kg^{-1}, i.v.). Baseline values in the two groups were not significantly different (P>0.05) in any of the tissues, including the liver, lungs and skin. Apart from decreases in liver conductance, no other changes in regional vascular conductances were noticed in the vehicle-treated group. BIBN4096BS produced small decreases in vascular conductance to liver, and with the highest dose (1000 μg.kg^{-1}) in lungs, adrenals, kidneys and spleen. Only the latter changes were significant when compared with the corresponding changes in the vehicle-treated animals.
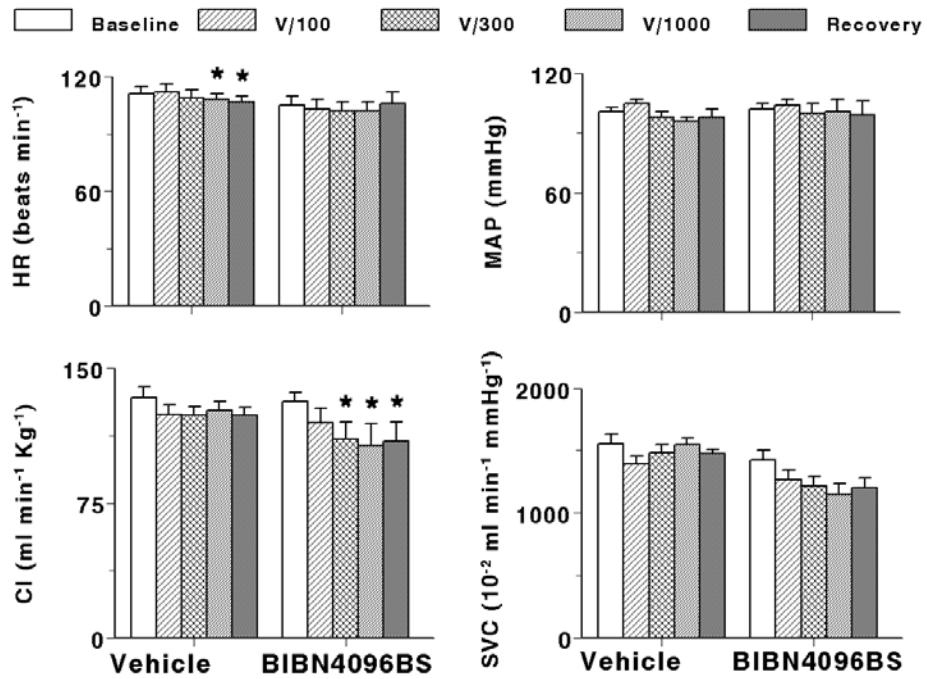
Figure 3.1. Heart rate (HR), mean arterial blood pressure (MAP), cardiac index (CI) and systemic vascular conductance (SVC) measured at baseline, after i.v. treatments with either vehicle (V, three times 5 ml; n=6) or BIBN4096BS (BIBN; 100, 300 and 1000 µg.kg^{-1}; n=7) and after 40 min recovery. All values are presented as mean±S.E.Mean. *, P<0.05 vs. baseline. The changes after BIBN4096BS are not significantly different from those in the corresponding vehicle group.
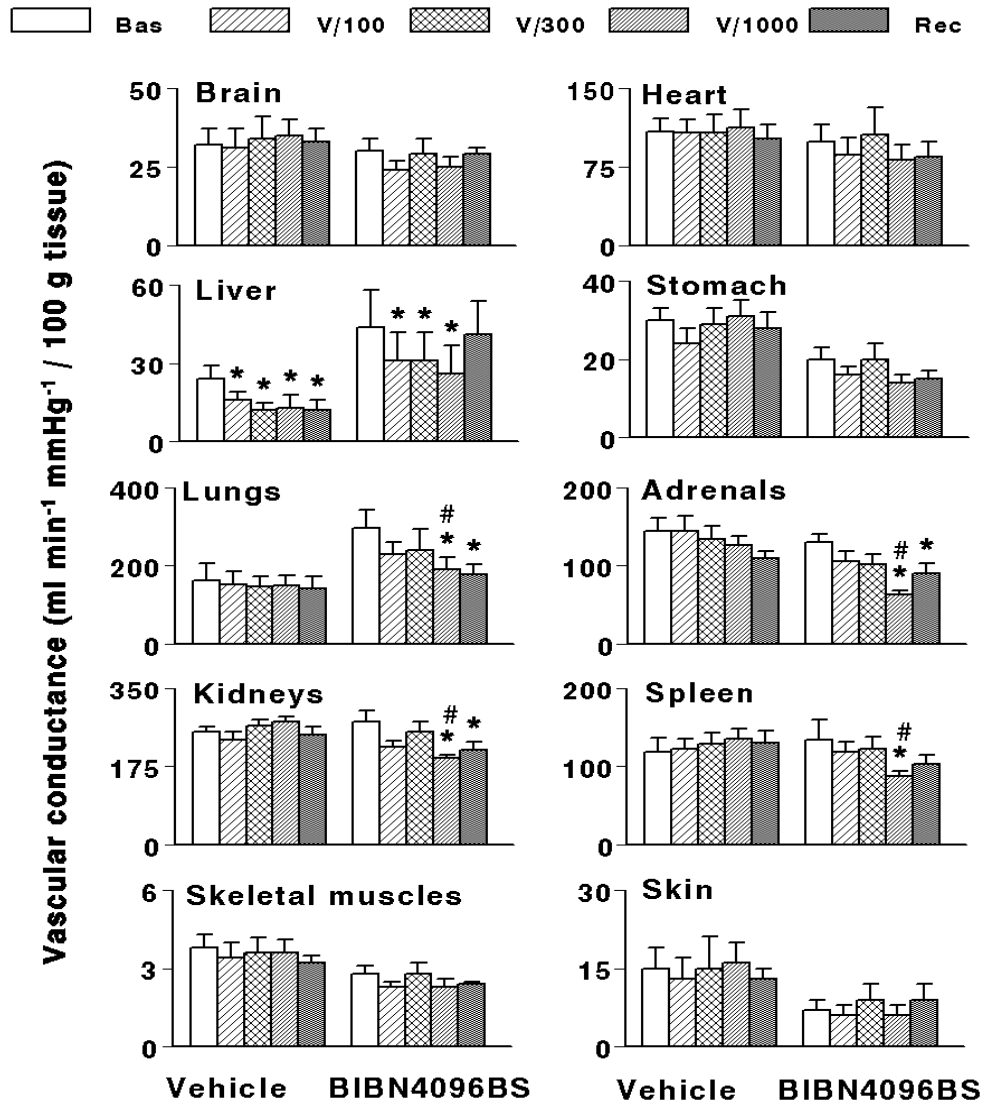
Figure 3.2. Regional vascular conductances at baseline (Bas), after i.v. treatments with either vehicle (V, three times 5 ml; n=6) or BIBN4096BS (100, 300, and 1000 µg.kg^{-1}, i.v.; n=6) and after 40 min recovery (Rec). All values are presented as mean±s.e.mean. *, $P<0.05$ vs. baseline. #, $P<0.05$ vs. the corresponding change in animals treated with vehicle.

3.3.2   Effect of BIBN4096BS on the haemodynamic responses to i.c. infusions of α-CGRP

BASELINE VALUES

Baseline values in anaesthetised pigs (n=13) were: heart rate, 129±5 beats.min^{-1}; mean arterial blood pressure, 122±4 mmHg; carotid flow pulse, 1.7±0.1 arbitrary units (a.u.); total carotid blood flow, 67±7 ml.min^{-1}; total carotid vascular conductance, 56±5 10^{-2} ml.min^{-1}.mmHg^{-1} and A-V SO$_2$ difference, 26±3%. Baseline values in the two groups of animals (vehicle and BIBN4096BS) did not differ significantly.

SYSTEMIC AND CAROTID HAEMODYNAMIC RESPONSES

Figure 3.3 shows the original tracings illustrating the systemic (blood pressure and heart rate) and carotid (flow pulse and total carotid blood flow) haemodynamic responses in anaesthetised pigs obtained with α-CGRP (10, 30 and 100 pmol.kg^{-1}.min^{-1}, i.c.) before and after i.v. treatments with three doses of vehicle (5 ml each time; upper panel) or BIBN4096BS (100, 300 and 1000 μg.kg^{-1}; lower panel). The infusions of α-CGRP did not affect heart rate, but decreased arterial blood pressure and increased carotid flow pulse and blood flow. These changes were accompanied by a redness of head skin and ears on the side of infusion (not shown in the Figure). The effects of α-CGRP were clearly attenuated in the animals receiving BIBN4096BS, but not in the ones treated with vehicle.

The effects of α-CGRP (10, 30 and 100 pmol.kg^{-1}.min^{-1}, i.c.) in the animals treated with vehicle or BIBN4096BS (100, 300 and 1000 μg.kg^{-1}, i.v.) were quantified as percent changes from baseline values (Figure 3.4). In both groups, infusions of α-CGRP before treatments with vehicle or BIBN4096 (control infusions) produced dose-dependent decreases in mean arterial blood pressure and increases in total carotid blood flow (data not shown) and conductance; heart rate was not affected (data not shown). These responses to α-CGRP remained unaffected after vehicle, but, in contrast, were dose-dependently antagonised by BIBN4096BS (Figure 3.4).

As shown in Figure 3.5, infusions of α-CGRP (100 pmol.kg^{-1}.min^{-1}, i.c.) clearly increased carotid blood flow (depicted as the maximum changes) and carotid blood flow pulsations (compare baseline and control values). While there was little

change in animals treated with vehicle, BIBN4096BS (100, 300 and 1000 μg.kg^{-1}, i.v.) dose-dependently antagonised the responses to α-CGRP.

CHANGES IN THE A-V SO$_2$ DIFFERENCE

α-CGRP (100 pmol.kg^{-1}.min^{-1}, i.c.) produced a significant reduction in the A-V SO$_2$ difference in both groups of animals (Figure 3.6; compare baseline and control values). The response to CGRP remained largely unaffected after treatments with vehicle, but BIBN4096BS (100, 300 and 1000 μg.kg^{-1}, i.v.) dose-dependently blocked the reduction in the A-V SO$_2$ difference by α-CGRP. In fact, the CGRP-induced decrease in the A-V SO$_2$ difference was enhanced after the highest dose of BIBN4096BS (Figure 3.6).

α-CGRP (pmol.kg^{-1}.min^{-1}, i.c.)
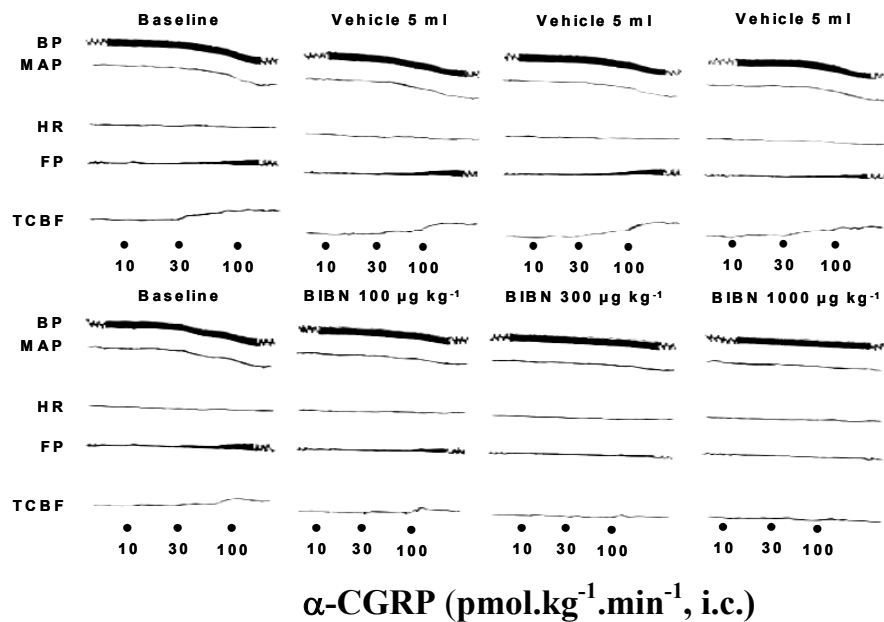
Figure 3.3. Original tracings from experiments in anaesthetised pigs illustrating systemic and carotid haemodynamic responses to infusions of α-CGRP ($\bullet$; 10, 30 or 100 pmol.kg^{-1}.min^{-1}, i.c.) given before and after i.v. treatments with either vehicle (three times 5 ml; *upper panel*) or BIBN4096BS (BIBN, 100, 300 and 1000 μg.kg^{-1}; *lower panel*). BP; systolic and diastolic arterial blood pressures; MAP, mean arterial blood pressure; HR, heart rate; FP, carotid blood flow pulse; TCBF, total carotid blood flow.
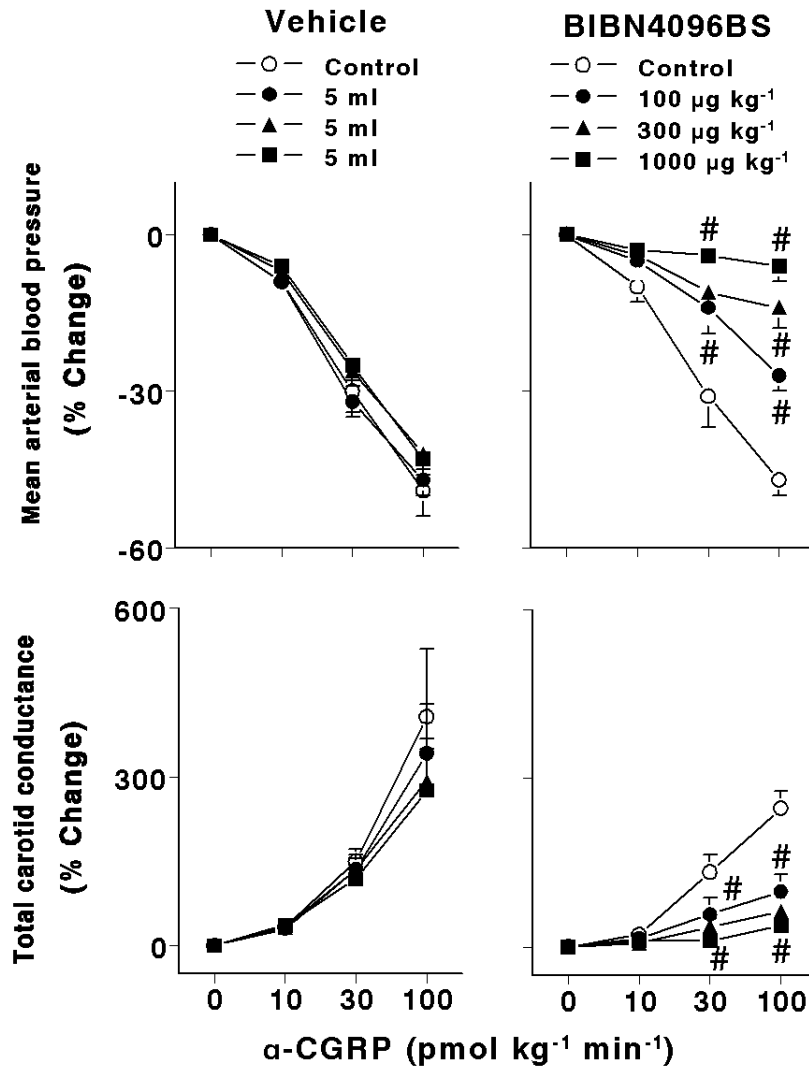
Figure 3.4. Changes in mean arterial blood pressure and total carotid vascular conductance from baseline values by i.c. infusion of α-CGRP in anaesthetised pigs given before (Control) and after i.v. treatments with vehicle (three times 5 ml; n=6) or BIBN4096BS (100, 300 and 1000 µg.kg^{-1}, n=7). All values are expressed as mean±s.e.mean. The two highest doses of α-CGRP significantly decreased mean arterial blood pressure and increased total carotid
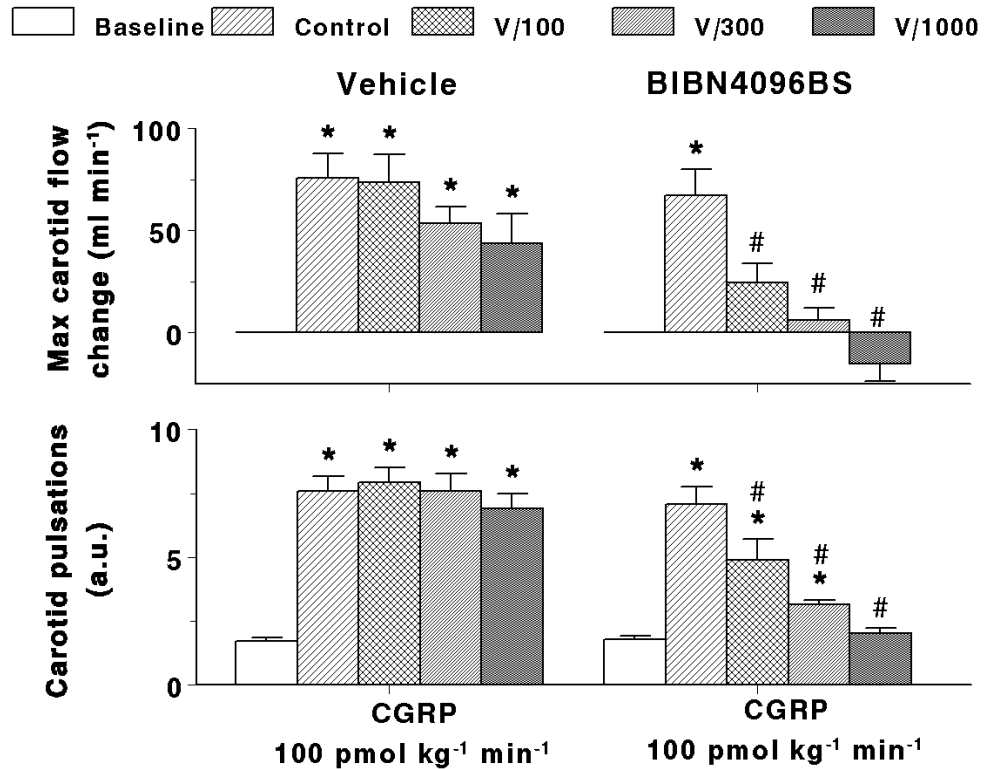
Figure 3.5. Maximum carotid blood flow changes and carotid blood flow pulsations measured at baseline and following infusions of α-CGRP (100 pmol.kg^{-1}.min^{-1}, i.c.) given in anaesthetised pigs before (Control) and after i.v. treatments with vehicle (V, 5 ml three times; n=6) or BIBN4096BS (100, 300 and 1000 μg.kg^{-1}, n=7). All values are expressed as mean±s.e.mean. a.u., Arbitrary units. *, P < 0.05 vs. baseline values; #, P<0.05 vs. response after the corresponding volume of vehicle.
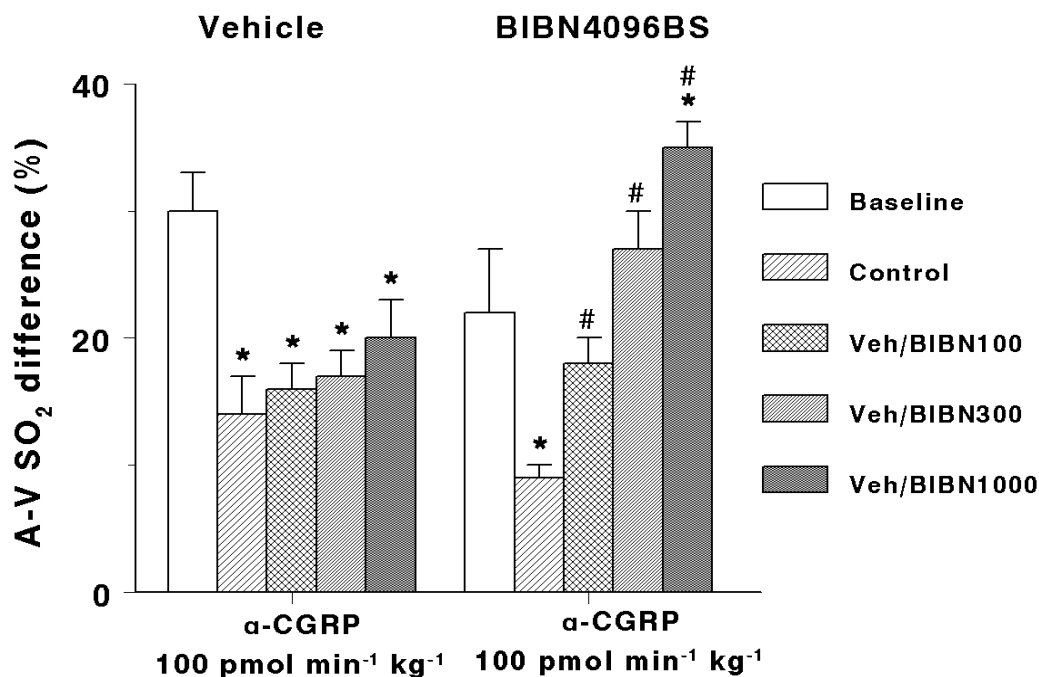
Figure 3.6. Differences between arterial and jugular venous oxygen saturations (A-V SO$_2$ difference) measured at baseline and after infusions of α-CGRP (100 pmol.kg^{-1}.min^{-1}, i.c.) given in anaesthetised pigs before (Control) and after i.v. treatments with vehicle (Veh, 5 ml three times; n=6) or BIBN4096BS (100, 300 and 1000 μg.kg^{-1}, n=7). All values are expressed as mean±s.e.mean. *, $P < 0.05$ vs. baseline values; #, $P < 0.05$ vs. response after the corresponding volume of vehicle.

3.4   Discussion

3.4.1   General

Undoubtedly, a remarkable progress has been achieved in acute antimigraine therapy (De Vries *et al.*, 1996a). Notwithstanding, the exact pathophysiological mechanisms underlying migraine remain unclear. There is, however, evidence supporting the involvement of the trigeminovascular system in migraine pathophysiology (Goadsby, 1997b; Goadsby, 1999; Hargreaves *et al.*, 1999; Williamson & Hargreaves, 2001). Thus, activation of the trigeminovascular system leads to neuropeptide release, including that of CGRP, and neurogenic dural vasodilatation (Williamson &

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Hargreaves, 2001). Of particular relevance is the finding that plasma concentration of CGRP is elevated during the headache phase of migraine, and this is normalised after treatment with sumatriptan (Goadsby *et al.*, 1990; Goadsby, 1997b; Goadsby, 1999). Hence, it is reasonable to assume that a potent CGRP receptor antagonist, such as BIBN4096BS (Doods *et al.*, 2000), might be useful in migraine therapy. BIBN4096BS behaves as a 'silent' competitive antagonist at CGRP receptors mediating relaxation of human temporal, cranial and coronary arteries (Edvinsson *et al.*, 2002; Moreno *et al.*, 2002; Verheggen *et al.*, 2002). The present study in anaesthetised pigs was designed: (i) to analyse, using BIBN4096BS, the potential role of endogenous CGRP in regulating vascular tone *in vivo*; and (ii) to investigate the effects of BIBN4096BS on the systemic and carotid haemodynamic responses produced by α-CGRP.

3.4.2   Systemic and regional haemodynamic effects of BIBN4096BS

It is well known that CGRP-immunoreactive nerve fibres are widely distributed in the cardiovascular system, with a higher preponderance in arteries than in veins (Bell & McDermott, 1996). CGRP decreases blood pressure and has positive inotropic and chronotropic effects on the heart (Wimalawansa, 1996), which are mainly mediated via $CGRP_1$ receptors (Bell & McDermott, 1996; Saetrum Opgaard *et al.*, 1999; Saetrum Opgaard *et al.*, 2000). Though CGRP has diverse biological actions within the cardiovascular system, our experiments showing few systemic haemodynamic changes with BIBN4096BS do not support a major role for CGRP in the regulation of cardiovascular function in the anaesthetised pig.

As far as regional haemodynamics is concerned, a moderate decrease (compared to vehicle) in vascular conductances in the lungs, adrenals, kidneys and spleen was observed with the highest dose ($1000 \, \mu g.kg^{-1}$) of BIBN4096BS (Figure 3.2). Similarly, renal vasoconstriction was noticed in conscious rats with a high ($300 \, nmol.kg^{-1}.min^{-1}$), but not with a low ($30 \, nmol.kg^{-1}.min^{-1}$) dose of CGRP- (8-37) (Gardiner *et al.*, 1990). Since both BIBN4096BS and $CGRP_{8-37}$ caused renal changes only in doses that were considerably higher than those needed for CGRP antagonism, it does not appear that endogenous CGRP regulates renal vascular tone. Also, Shen et al. (Wu *et al.*, 2001) recently reported that $30 \, \mu g.kg^{-1}.min^{-1}$ ($\sim 10 \, nmol.kg^{-1}.min^{-1}$) of CGRP-(8-37) which antagonised CGRP-induced

haemodynamic responses, caused little regional haemodynamic effects in conscious dogs as well as anaesthetised rats, thereby not supporting an important physiological role for endogenous CGRP in regulating vascular tone. Although we cannot rule out the involvement of CGRP in certain other circumstances, for example, cardiac preconditioning or coronary artery disease (Lu *et al.*, 1999; Peng *et al.*, 2000; Wu *et al.*, 2001), the present results imply cardiovascular safety of BIBN4096BS. Nevertheless, one will have to explore the role of CGRP in cardiovascular pathophysiology before establishing whether or not CGRP receptor antagonists are completely safe in patients afflicted with cardiovascular disorders.

3.4.3   CGRP-induced   haemodynamic   responses   and   antagonism   by BIBN4096BS

Activation of CGRP receptors elicits dilatation in different vascular beds in several species (Gardiner *et al.*, 1990; Van Gelderen *et al.*, 1995; Shen *et al.*, 2001). Consistent with these studies, our experiments show that i.c. infusions of α-CGRP produced a marked vasodilatation in the porcine carotid circulation, with accompanying fall in arterial blood pressure. The fact that the animals were systematically vagosympathectomised may explain why the hypotension was not accompanied by a baroreflex-mediated tachycardia, as reported earlier (Van Gelderen *et al.*, 1995). Interestingly, the ipsilateral skin redness, together with the marked decrease in A-V SO_2 difference by CGRP, indicates that porcine carotid arteriovenous anastomoses dilated in response to α-CGRP (Saxena, 1987). However, we previously reported that i.c. infusions of α-CGRP failed to increase porcine arteriovenous anastomotic blood flow, despite a marked increase in the total carotid and capillary blood flows (Van Gelderen *et al.*, 1995). Admittedly, arteriovenous anastomotic blood flow was not directly measured in these experiments, but we have recently observed that i.c. infusions capsaicin, which released CGRP, did increase carotid arteriovenous anastomotic blood flow with a concomitant decrease in the A-V SO_2 difference (Tom *et al.*, 2002). Thus, it appears that the discrepancy between the two investigations may be due to different anaesthetic regimens employed (pentobarbital and fentanyl/thiopental, respectively) and, particularly, the use of phenylephrine in the present experiments. Phenylephrine potently constricts arteriovenous anastomoses (Willems *et al.*, 1999).

In the present experimental study in anaesthetised pigs, BIBN4096BS proved to be an effective antagonist at the CGRP receptors mediating the systemic (hypotension) as well as the carotid (increased blood flow, pulsations and skin redness) haemodynamic responses to α-CGRP. The fact that BIBN4096BS also abolished α-CGRP-induced decreases in the A-V SO_2 difference suggests its action on carotid arteriovenous anastomoses; for further considerations, see Saxena (1987). Interestingly, BIBN4096BS also antagonised the capsaicin-induced increases in carotid arteriovenous anastomotic blood flow as well as decreases in the A-V SO_2 difference, but not the plasma CGRP concentrations (Kapoor *et al.*, 2003a).

One cannot be certain about the nature of CGRP receptors that mediate porcine carotid vascular responses, but cardiac inotropic and vasodilator responses are mediated predominantly by $CGRP_1$ receptors (Saetrum Opgaard *et al.*, 1999), where BIBN4096BS has a very high affinity (Doods *et al.*, 2000; Poyner & Marshall, 2001).

3.4.4   Potential therapeutic efficacy of BIBN4096BS in the treatment migraine

Considering that plasma CGRP levels are elevated during the headache phase of migraine (Goadsby, 1997b) and that BIBN4096BS dose-dependently blocked α-CGRP-induced carotid haemodynamic responses, it is likely that BIBN4096BS may be effective in migraine. The compound is presently under clinical investigation for the acute treatment of migraine and the results are awaited with great interest.

In conclusion, our study clearly demonstrates that BIBN4096BS is an effective antagonist at vascular CGRP receptors in anaesthetised pigs, but has little haemodynamic effects of its own, a finding that negates a major physiological role for CGRP in cardiovascular regulation. The potent blockade of the carotid haemodynamic effects of CGRP does suggest that BIBN4096BS may be effective in migraine treatment.

CHAPTER 4

Effects of sumatriptan on capsaicin-induced carotid haemodynamic changes and CGRP release in anaesthetised pigs

4   Effects of sumatriptan on capsaicin-induced carotid haemodynamic changes and CGRP release in anaesthetised pigs

Abstract: It is suggested that during a migraine attack capsaicin-sensitive trigeminal sensory nerves release calcitonin gene related peptide (CGRP), resulting in cranial vasodilatation and central nociception. Hence, inhibition of trigeminal CGRP release may prevent the above vasodilatation and, accordingly, abort migraine headache. Therefore, this study investigated the effects of sumatriptan (100 and 300 μg kg^{-1}, i.v.) on capsaicin-induced carotid haemodynamic changes and on CGRP release. Intracarotid (i.c.) infusions of capsaicin (10 μg kg^{-1} min^{-1}, i.c.) increased total carotid, arteriovenous anastomotic and capillary conductances as well as carotid pulsations, but decreased the difference between arterial and jugular venous oxygen saturations. Except for some attenuation of arteriovenous anastomotic changes, the capsaicin-induced responses were not affected by sumatriptan. Moreover, i.c. infusions of capsaicin (0.3, 1, 3 and 10 μg kg^{-1} min^{-1}, i.c.) dose-dependently increased the jugular venous plasma concentrations of CGRP, which also remained unaffected by sumatriptan. The above results support the contention that the therapeutic action of sumatriptan is mainly due to cranial vasoconstriction rather than trigeminal (CGRP release) inhibition.

4.1   Introduction

Migraine is a neurovascular disorder characterised by vasodilatation of cranial blood vessels with activation of perivascular trigeminal sensory nerves (Edvinsson, 2003). Thus, activation of trigeminal sensory nerves results in the release of several neuropeptides, including neuropeptide Y, vasoactive intestinal peptide, substance P and calcitonin gene related peptide (CGRP). Interestingly, plasma concentrations of CGRP, but not of other neuropeptides, are elevated during the headache phase of migraine and these levels are normalised by triptans in parallel with alleviation of headache (Goadsby *et al.*, 2002b). CGRP, the most potent endogenous vasodilator described thus far, is predominantly located on sensory neurons and perivascular nerves surrounding blood vessels, where it is co-localised with other vasoactive neuropeptides, such as substance P and neurokinin A (Brain *et al.*, 1985; van Rossum *et al.*, 1997). In view that an increase in circulating CGRP levels is considered as a biological marker for migraine headache, it is reasonable to assume that a substance capable of inhibiting the release of CGRP from trigeminal sensory nerves may be effective in migraine therapy (Goadsby *et al.*, 2002b).

CGRP can be released from the sensory nerves by electrical or chemical (capsaicin) stimuli (Edvinsson, 2001b; Hou *et al.*, 2002). In this respect, electrical stimulation of trigeminal sensory nerves evokes the release of CGRP in the cranial venous blood of rats and cats, which was attenuated by sumatriptan (Goadsby & Edvinsson, 1993; Buzzi *et al.*, 1995). However, to the best of our knowledge, there is no comprehensive *in vivo* evidence to show that capsaicin-induced CGRP release is inhibited by triptans. Therefore, the main objective of this study in anaesthetised vagosympathectomised pigs was to investigate the effects of sumatriptan on capsaicin-induced: (i) carotid haemodynamic changes, and (ii) increase in plasma CGRP release.

4.2   Materials and methods

4.2.1   General

After an overnight fast, 28 domestic pigs (Yorkshire x Landrace, female, 10-14 kg), divided into two groups (n=15 and n=13 for vehicle and sumatriptan, respectively), were sedated with azaperone (120 mg, i.m.), midazolam hydrochloride (10 mg, i.m.) and then anaesthetised with sodium pentobarbital (600 mg, i.v.). After tracheal intubation, the animals were connected to a respirator (BEAR 2E, BeMeds AG, Baar, Switzerland) for intermittent positive pressure ventilation with a mixture of room air and oxygen. Respiratory rate, tidal volume and oxygen supply were adjusted to keep arterial blood gas values within physiological limits (pH: 7.35-7.48, pCO_2: 35-48 mmHg, pO_2: 100-120 mmHg). Anaesthesia was maintained with a continuous i.v. infusion of sodium pentobarbital (12-20 $mg\,kg^{-1}\,h^{-1}$). This anaesthetic regimen, together with bilateral vagosympathectomy (see below), increases heart rate and markedly dilates carotid arterioles and arteriovenous anastomoses. Consequently, carotid blood flow, particularly its arteriovenous anastomotic fraction, is considerably higher in these pigs than in conscious or thiopental-anaesthetised pigs (Den Boer *et al.*, 1993).

Heart rate was measured with a tachograph (CRW, Erasmus University, Rotterdam, The Netherlands) triggered by electrocardiogram signals. Both common carotid arteries were dissected free and the accompanying vagosympathetic trunks were cut between two ligatures to prevent any possible influence via baroreceptor reflexes on the carotid vascular responses produced by capsaicin. Pulsatile and mean

carotid blood flows were measured in the right common carotid artery with a flow probe (internal diameter: 2.5 mm) connected to a sine-wave electromagnetic flow meter (Transflow 601-system, Skalar, Delft, The Netherlands). The amplitude of carotid blood flow signals provided an index of carotid flow pulse. Subsequently, three hub-less needles, connected to a polyethylene tube, were inserted into the right common carotid artery for the administration of capsaicin, radioactive microspheres and the α_1-adrenoceptor agonist phenylephrine. The use of phenylephrine is necessitated by the fact that the carotid arterioles and arteriovenous anastomoses are in a dilated state under the present anaesthetic regimen (Den Boer *et al.*, 1993); therefore, to study the effects of vasodilator agents (in the present case, capsaicin), one has to constrict these shunt vessels first. As described earlier (Willems *et al.*, 1999), phenylephrine decreases the total carotid conductance exclusively by constricting carotid arteriovenous anastomoses, which results in an increase in the difference between arterial and jugular venous oxygen saturations (A-V SO$_2$ difference) (Saxena, 1987).

Lastly, catheters were placed in: (i) the right external jugular vein for the withdrawal of venous blood samples to measure blood gases (ABL-510; Radiometer, Copenhagen, Denmark) and plasma concentrations of CGRP (see below); (ii) the inferior vena cava (via the left femoral vein) for the administration of vehicle or sumatriptan; and (iii) the aortic arch (via the left femoral artery) for the measurement of arterial blood pressure (Combitrans disposable pressure transducer; Braun, Melsungen, Germany) as well as withdrawal of arterial blood samples to measure blood gases.

Heart rate and systolic, diastolic and mean arterial blood pressures as well as mean and pulsatile carotid artery blood flows were continuously monitored on a polygraph (CRW, Erasmus University, Rotterdam, The Netherlands). Vascular conductances were calculated by dividing the respective blood flows (ml min^{-1}) by mean arterial blood pressure (mmHg), multiplied by one hundred and expressed as 10^{-2} ml min^{-1} mmHg^{-1}. During the experiment, body temperature was maintained at $37\pm1°C$ by a heating pad and each animal was infused with physiological saline to compensate for fluid losses.

4.2.2 Distribution of carotid blood flow

The distribution of common carotid blood flow into tissue (capillary) and arteriovenous anastomotic fractions was determined in 14 pigs later receiving vehicle (n=8) or sumatriptan (n=6) with radioactive microspheres (diameter: 15.5±0.1 μm; S.D.), labelled with ^{141}Ce, ^{103}Ru, ^{95}Nb or ^{46}Sc (NEN Dupont, Boston, USA). For each measurement, a suspension of about 200,000 microspheres, labelled with one of these isotopes, was mixed and injected into the right carotid artery. At the end of the experiment, the animal was killed using an overdose of sodium pentobarbital and the heart, kidneys, lungs and different cranial tissues were dissected out, weighed and put in vials. The radioactivity in these vials was counted for 5 min in a γ-scintillation counter (Packard, Minaxi autogamma 5000 for 5 min), using suitable windows for discriminating the different isotopes (^{141}Ce: 120-167 KeV, ^{103}Ru: 450-548 KeV, ^{95}Nb: 706-829 KeV and ^{46}Sc: 830-965 KeV). All data were processed by a set of specially designed computer programs (Saxena *et al.*, 1980).

The distribution of total carotid blood flow to the different tissues (Q_{tis}) was calculated by the formula: $Q_{tis} = (I_{tis}/I_{total})$ x $Q_{carotid}$, where I_{tis} is tissue radioactivity, I_{total} is the total radioactivity injected and $Q_{carotid}$ is the total common carotid blood flow at the time of microsphere injection. Since little or no radioactivity was detected in the heart or kidneys, it can be assumed that all microspheres trapped in lungs reach this tissue from the venous side after escaping via carotid arteriovenous anastomoses. Therefore, the amount of radioactivity in the lungs can be used as an index of the arteriovenous anastomotic fraction of carotid blood flow (Saxena *et al.*, 1980; Saxena & Verdouw, 1982).

4.2.3 Determination of plasma levels of CGRP

Jugular venous blood samples were obtained from the 28 pigs, receiving either vehicle (n=15) or sumatriptan (n=13). Fourteen of these animals (8 and 6 animals from vehicle and sumatriptan groups, respectively) were used for carotid haemodynamic experiments, while the other fourteen were separate experiments using the same protocol, except that the radioactive microspheres were not used. Blood was transferred immediately into a polypropylene tube containing ethylene dinitro-tetraacetic acid (1 mg ml^{-1} of blood) and aprotinin (500 KIU ml^{-1} of blood). Aprotinin was used to inhibit endogenous plasma proteases, since our unpublished studies have

shown that CGRP is not detectable in biological samples without aprotinin. After centrifugation at 1600 g for 15 min, plasma samples were coded and stored at -80°C until CGRP measurements were performed. The person measuring CGRP concentrations remained blind to the treatments, until all data had been collated.

CGRP was extracted from plasma using a C_{18} SEP-COLUMN, dried by lypholisation, and measured by radioimmunoassay (Dwenger, 1984), as per protocol of the Peninsula Laboratories, Inc. (Belmont, CA, U.S.A.). The recovery of CGRP from the extraction procedure was ascertained by assaying control samples paired with the same sample spiked with known quantities of CGRP. The column recovery values were 85, 79, 81, 89 and 92% (mean=85.2; standard deviation=5.4; coefficient of variation=6.3%). The CGRP concentrations measured in the actual samples were, however, not corrected for the loss in the extraction procedure.

4.2.4   Experimental protocol

Following surgery and after the haemodynamic condition of the animals (n=28) had been stable for 15-20 min (heart rate: 109±3 beats min^{-1}; mean arterial blood pressure: 101±2 mmHg; mean carotid blood flow: 108±5 ml min^{-1}; and A-V SO_2 difference: 7.6±1.3%) phenylephrine was infused into the right common carotid artery at a rate of 10 μg kg^{-1} min^{-1} for 10 min, followed by 3-6 μg kg^{-1} min^{-1} throughout the rest of the experiment to maintain carotid blood flow at a constant low level. The latter dose of phenylephrine was chosen so that the external jugular venous oxygen saturation was between 60-70% and mean carotid blood flow was about 40% of the original value. After a period during which the haemodynamic variables remained constant for at least 60 min (heart rate: 151±4 beats min^{-1}; mean arterial blood pressure: 110±2 mmHg; mean carotid blood flow: 63±5 ml min^{-1}; and A-V SO_2 difference: 23±1.7%; n=28), the animals received consecutive infusions (0.15, 0.45, 1.5 and 4.5 ml, i.c. for 3 min each) of capsaicin vehicle (see Compounds and kits section). It is important to mention that the vehicle of capsaicin was devoid of any systemic and carotid haemodynamic responses (see Results section).

Five to ten min after the last infusion of capsaicin vehicle, blood samples were obtained for the measurements of blood gases and CGRP concentrations; moreover, the values of heart rate, mean arterial blood pressure and total carotid blood flow and conductance were collated (baseline values; 15 and 13 pigs for vehicle and

sumatriptan, respectively). In 14 out of the 28 pigs (8 for vehicle and 6 for sumatriptan) the first batch of radioactive microspheres was injected for determining the baseline distribution of carotid blood flow. The animals then received 4 consecutive infusions of capsaicin (0.3, 1, 3 and 10 μg kg^{-1} min^{-1}, i.c. for 3 min each) and heart rate, arterial blood pressure and total carotid blood flow were determined at the end of each infusion. In addition, after the last infusion of capsaicin (10 μg kg^{-1} min^{-1}), blood gases, plasma CGRP concentration and carotid blood flow distribution were measured as described above (control values). Subsequently, a recovery period of 20 min was allowed until all haemodynamic parameters had returned to baseline levels. At this point, the animals were divided into two groups, which were treated with i.v. infusions (rate: 0.5 ml min^{-1} for 10 min) of either vehicle (two times 5 ml of acidified distilled water; n=15) or sumatriptan (100 and subsequently 300 μg kg^{-1}; n=13). Ten min after each infusion, capsaicin was given and the haemodynamic and biochemical variables were measured again, as described above.

In the remaining fourteen animals, subdivided into two subgroups (n=7 each; for vehicle and sumatriptan, respectively), apart from determining the variables described above, venous blood samples (for plasma CGRP concentration) were withdrawn after each dose of capsaicin (0.3, 1 and 3 and 10 μg kg^{-1} min^{-1}) given before and after treatment with vehicle (n=7) or sumatriptan (300 μg kg^{-1}; n=7).

4.2.5   Data presentation and statistical analysis

All data are presented as mean±s.e.mean. The statistical analysis was performed using the SPSS package for windows (version 10.0; SPSS Inc., Chicago, IL, USA). The significance of changes within one group (vehicle or sumatriptan) was analysed with repeated-measures ANOVA, followed by Greenhouse-Geisser correction for serial autocorrelation (Ludbrook, 1994) and Bonferroni correction for multiple comparisons (Overall & Doyle, 1996). The significance of the between-group changes (vehicle vs. sumatriptan treatments) was first analysed with repeated-measures ANOVA, including baseline measurements as a covariate (Overall & Doyle, 1994). If the two groups differed significantly, pairwise comparisons between the corresponding values in the vehicle- and sumatriptan-treated groups were performed

using univariate analysis (Overall & Atlas, 1999), followed by Bonferroni correction. Statistical significance was accepted at P<0.05 (two-tailed).

4.2.6   Ethical approval

The Ethics Committee of the Erasmus MC, Rotterdam, dealing with the use of animals in scientific experiments, approved the protocols for this investigation.

4.2.7   Compounds and kits

The following compounds were used: aprotinin (5850 KIU mg^{-1}; Roth, Karlsruhe, Germany), azaperone (Stresnil$^®$; Janssen Pharmaceuticals, Beerse, Belgium), sumatriptan succinate (gift from Dr. H.E. Connor, Glaxo Group Research, Stevenage, Hertfordshire, UK), capsaicin, tween 80, ethanol and phenylephrine hydrochloride (all from Sigma-Aldrich Chemie b.v., Zwijndrecht, The Netherlands), ethylene dinitro-tetraacetic acid (Merck, Darmstadt, Germany), heparin sodium (to prevent blood clotting in catheters; Leo Pharmaceutical Products, Weesp, The Netherlands), midazolam hydrochloride (Dormicum$^®$; Hoffmann La Roche b.v., Mijdrecht, The Netherlands) and sodium pentobarbital (Sanofi Sante b.v., Maasluis, The Netherlands).   The radioimmunoassay kit for CGRP was purchased from Peninsula Laboratories, Inc. (Belmont, CA, U.S.A.).

Capsaicin was initially dissolved in tween 80, ethanol and physiological saline in the ratio of 0.5:1:8.5 ml, respectively.   Phenylephrine was dissolved in distilled water, while sumatriptan was dissolved in physiological saline.

4.3   Results

4.3.1   Baseline values

Baseline values (i.e.,, after capsaicin vehicle infusion) in the 28 pigs used were: heart rate, 133±3 beats min^{-1}; mean arterial blood pressure, 108±2 mmHg; total carotid blood flow, 47±4 ml min^{-1}; total carotid conductance, 44±3 10^{-2} ml min^{-1} mmHg^{-1}; A-V SO$_2$ difference, 36±2%; and plasma CGRP concentration, 13±1 pg ml^{-1}.   No significant difference in the baseline values were found between the two groups that subsequently received vehicle (n=15) or sumatriptan (n=13).

4.3.2   Effect of different doses of capsaicin on heart rate, blood pressure and total carotid blood flow and conductance

Figure 4.1 depicts heart rate, mean arterial blood pressure and total carotid blood flow and conductance changes produced by different doses of capsaicin (0.3, 1, 3 and 10 μg kg^{-1} min^{-1}, i.c.) before (control response) and after treatment with sumatriptan (100 and 300 μg kg^{-1}, i.v.) or the corresponding volumes of vehicle. In both groups, capsaicin infusion dose-dependently increased the heart rate (last two doses), mean arterial blood pressure and total carotid blood flow and conductance. These changes to capsaicin remained essentially unmodified following vehicle or sumatriptan treatment. However, a small, but significant, attenuation in capsaicin-induced increase in total carotid conductance was observed following the highest dose of sumatriptan treatment (300 μg kg^{-1}, i.v.; $P < 0.05$; Figure 4.1).
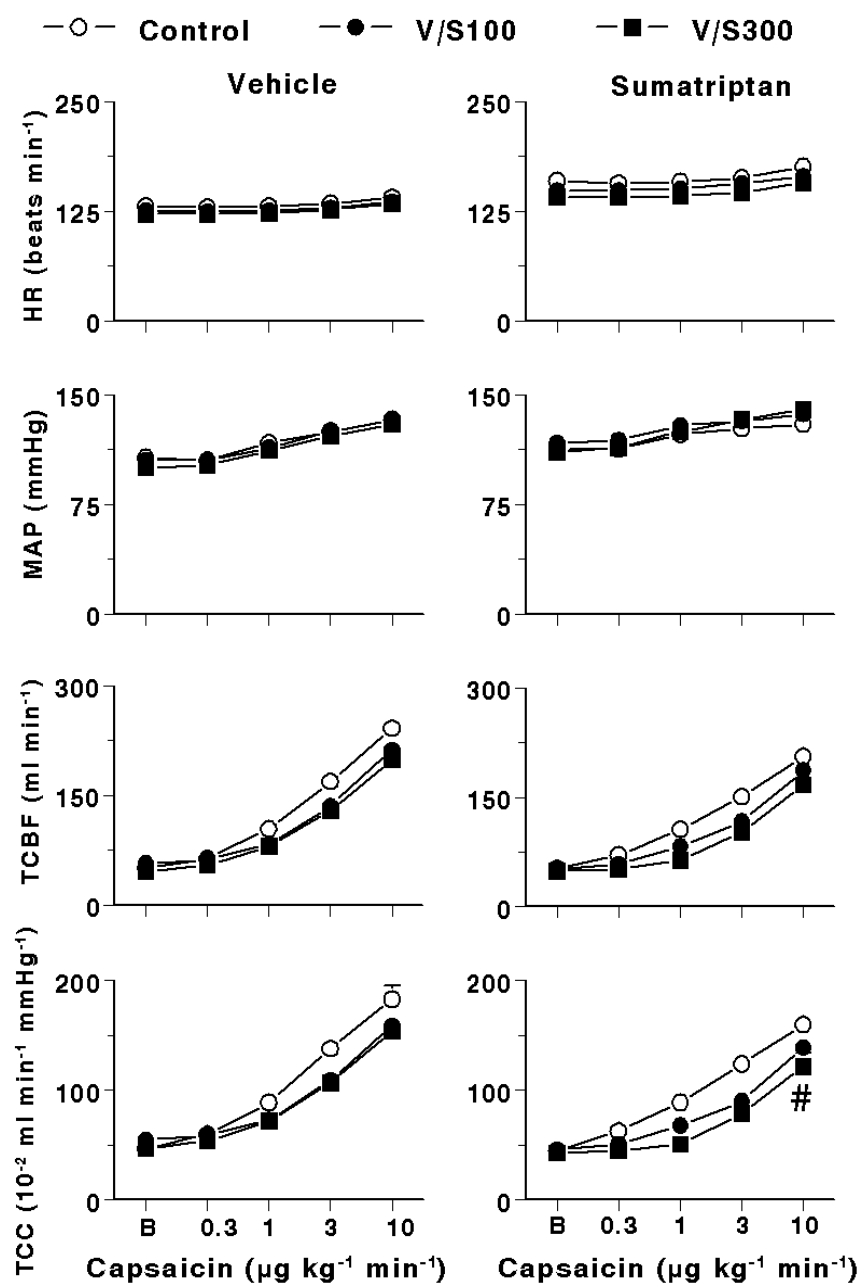
Figure 4.1. Heart rate (HR), mean arterial blood pressure (MAP), total carotid blood flow (TCBF) and total carotid vascular conductance (TCC) values at baseline (B) and following infusions of capsaicin (0.3, 1, 3, 10 $\mu g\,kg^{-1}\,min^{-1}$, i.c.) in anaesthetised pigs before (Control) and after i.v. administrations of vehicle (V, 5 ml two times; n=15) or sumatriptan (S100 and S300, 100 and 300 $\mu g\,kg^{-1}$, respectively; n=13). All values are expressed as mean±s.e.mean. #, P<0.05 vs. response after the corresponding volume of vehicle.

4.3.3   Capsaicin-induced carotid haemodynamic changes

The effects of capsaicin (10 µg kg^{-1} min^{-1}, i.c.) on carotid haemodynamics were investigated in detail in animals receiving vehicle or sumatriptan.

CAROTID BLOOD FLOW, CONDUCTANCE AND PULSATIONS

The effects of capsaicin (10 µg kg^{-1} min^{-1}, i.c.) on total carotid blood flow and conductance (depicted as maximum absolute changes) as well as pulsations (represented as arbitrary units; a.u.), before (control response) and after treatment with sumatriptan (100 and 300 µg kg^{-1} min^{-1}, i.v.) or the corresponding volumes of vehicle are shown in Figure 4.2. In both treatment groups, the capsaicin infusion significantly increased the carotid blood flow and conductance as well as pulsations. While vehicle and sumatriptan (100 µg kg^{-1}, i.v.) were devoid of any significant effect on capsaicin-induced increases in carotid haemodynamics, a small, but significant, decrease in the carotid vascular conductance was observed in the animals treated with the highest dose of sumatriptan (300 µg kg^{-1}, i.v.; $P<0.05$; Figure 4.2).

FRACTIONATION OF CAROTID VASCULAR CONDUCTANCE

As shown in Figure 4.3, the capsaicin infusion significantly increased total carotid, arteriovenous anastomotic and capillary conductances.   The capsaicin-induced increases in conductances from baseline values (maximal percent changes) were, respectively: total carotid, 329±39; arteriovenous anastomoses, 554±406; and capillary fraction, 340±30. While vehicle as well as sumatriptan treatment did not affect capsaicin-induced changes in total carotid and capillary fractions, the increase in arteriovenous anastomotic conductance was markedly attenuated by sumatriptan. Moreover, following the highest dose of sumatriptan (300 µg kg^{-1}, i.v.), a small decrease in capsaicin-induced increase in total carotid conductance was observed (#, $P<0.05$; see Figures 4.1 and 4.3).

Furthermore, capsaicin infusion significantly increased the vascular conductance to the different cranial tissues, including skin, ear, skeletal muscles, fat, bone, salivary glands, eye, tongue and dura mater, but not that to brain.   These vasodilator responses to capsaicin remained unaltered after sumatriptan (100 and 300 µg kg^{-1}, i.v.) or the corresponding volumes of vehicle (Figure 4.4).

4.3.4 Difference between arterial and jugular venous oxygen saturations (A-V SO$_2$ difference)

Consistent with the increase in arteriovenous anastomotic conductance, capsaicin infusion (10 μg kg^{-1} min^{-1}, i.c.) significantly decreased the A-V SO$_2$ difference from baseline values from 39±4% to 4±0.6% (control response; n=28). This response to capsaicin remained unaffected after treatment with vehicle or sumatriptan (Figure 4.5).
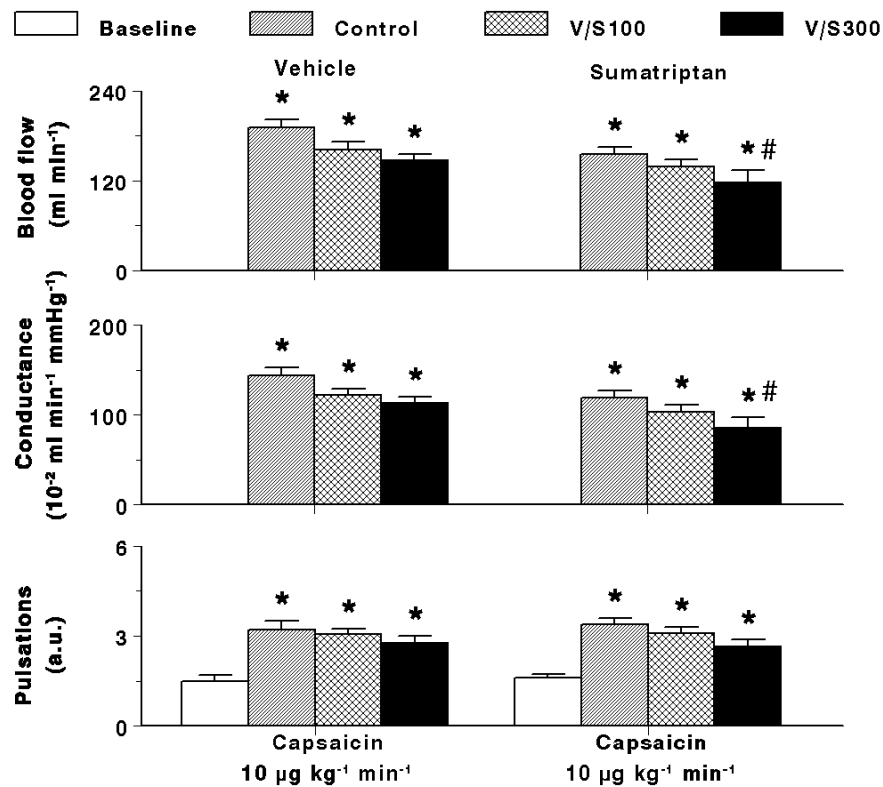
Figure 4.2. Maximum changes in carotid blood flow, vascular conductance and pulsations measured at baseline and following infusions of capsaicin (10 μg kg^{-1} min^{-1}, i.c.) given in anaesthetised pigs before (Control) and after i.v. administrations of vehicle (V, 5 ml two times; n=15) or sumatriptan (S100 and S300, 100 and 300 μg kg^{-1}, respectively; n=13). All values are expressed as mean±s.e.mean. a.u., Arbitrary units. *, P < 0.05 vs. baseline values; #, P<0.05 vs. response after the corresponding volume of vehicle.
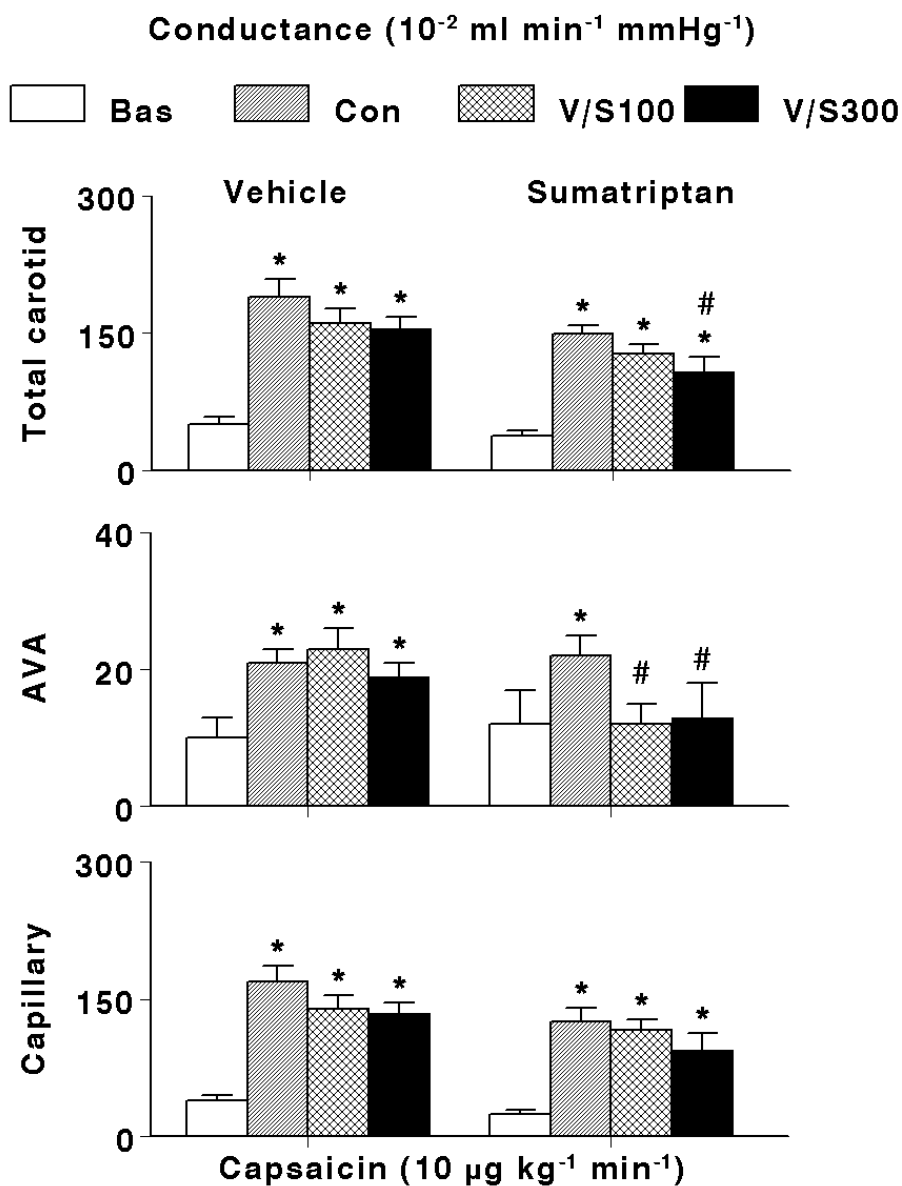
Figure 4.3. Total carotid, arteriovenous anastomotic (AVA) and capillary vascular conductances measured at baseline and following infusions of capsaicin ($10\,\mu g\,kg^{-1}\,min^{-1}$, i.c.) given in anaesthetised pigs before (Control) and after i.v. administrations of vehicle (V, 5 ml, two times) or sumatriptan (S100 and S300, 100 and 300 $\mu g\,kg^{-1}$, respectively). All values are expressed as mean±s.e.mean. *, P < 0.05 vs. baseline values; #, P<0.05 vs. response after the corresponding volume of vehicle.
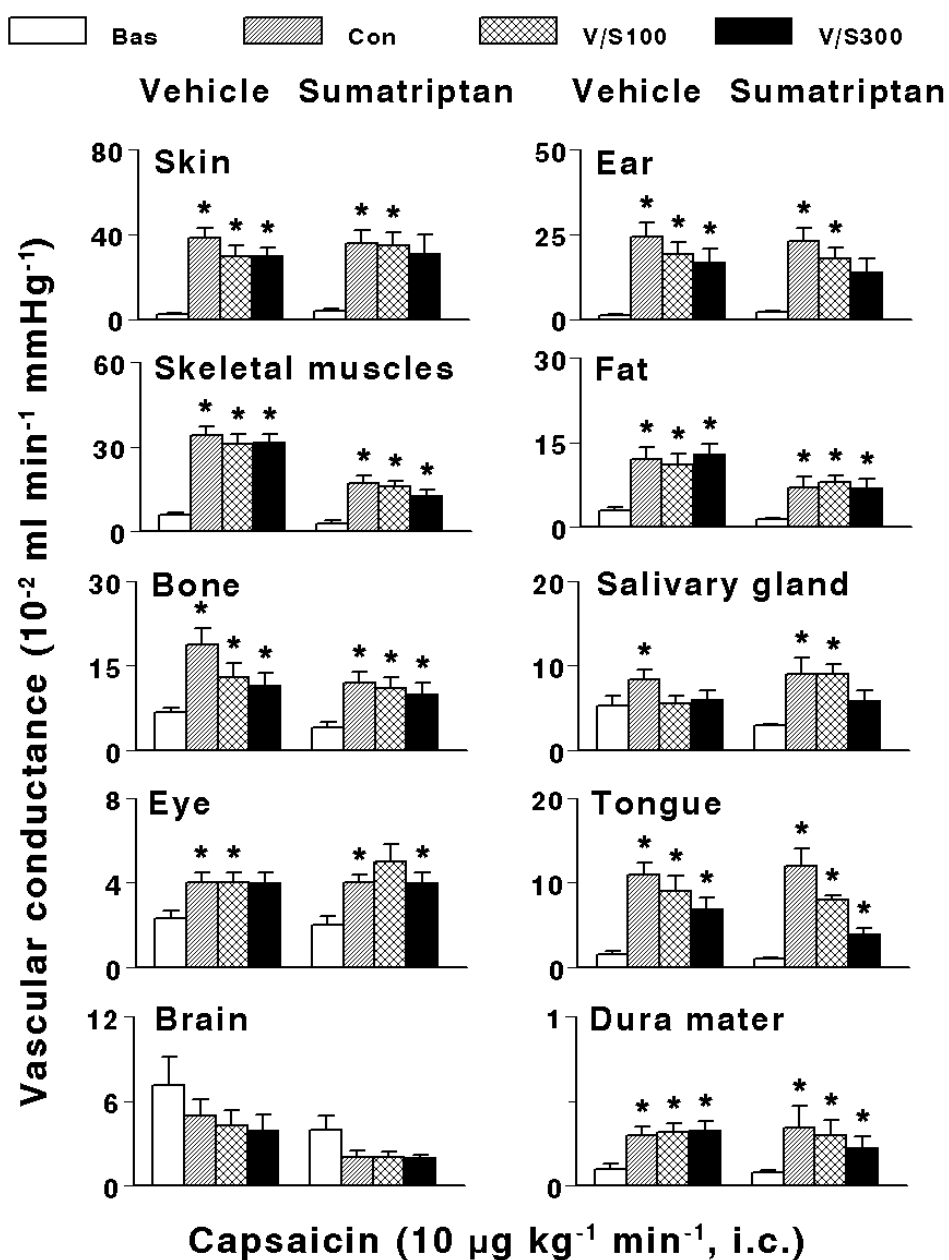
Figure 4.4. Distribution of carotid vascular conductances to head tissues measured at baseline (Bas) and following infusions of capsaicin (10 $\mu g\,kg^{-1}\,min^{-1}$, i.c.) given in anaesthetised pigs before (Con) and after i.v. administrations of vehicle (V, 5 ml, two times; n=8) or sumatriptan (S100 and S300, 100 and 300 $\mu g\,kg^{-1}$, respectively; n=6). All values are expressed as mean±s.e.mean. *, P < 0.05 vs. baseline values.
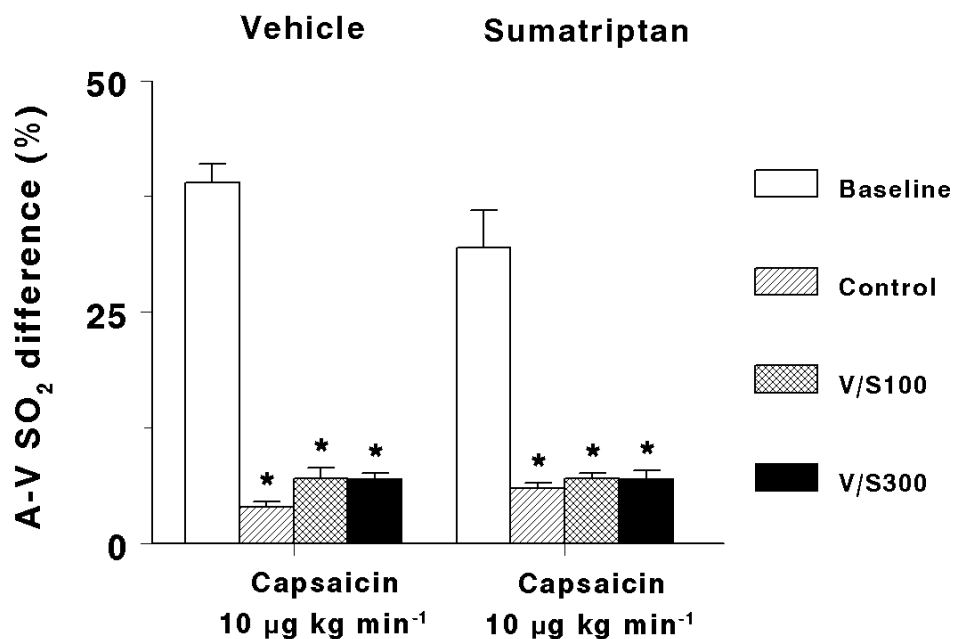
Figure 4.5. Differences between arterial and jugular venous oxygen saturations (A-V SO_2 difference) measured at baseline and after infusions of capsaicin (10 µg kg^{-1} min^{-1}, i.c.) given in anaesthetised pigs before (Control) and after i.v. administrations of vehicle (V, 5 ml, two times; n=15) or sumatriptan (S100 and S300, 100 and 300 µg kg^{-1}, respectively; n=13). All values are expressed as mean±s.e.mean. *, P < 0.05 vs. baseline values.

4.3.5 Capsaicin-induced jugular venous plasma concentration of CGRP

Figure 4.6 depicts the plasma CGRP concentrations at baseline (i.e.,, after capsaicin vehicle infusion) and following capsaicin infusion (10 µg kg^{-1} min^{-1}, i.c.) before (control response) and after sumatriptan (100 and 300 µg kg^{-1}, i.v.) or the corresponding volumes of vehicle. The capsaicin infusion significantly increased plasma CGRP concentrations from a baseline value of 11±1 pg ml^{-1} to 28±4 pg ml^{-1} (maximal percent change from baseline: 155±38). These responses to capsaicin were not attenuated by either vehicle or sumatriptan.

Furthermore, capsaicin infusions (0.3, 1,3 and 10 µg kg^{-1} min^{-1}, i.c.) dose-dependently increased plasma CGRP concentrations (a significant increase was observed during the last two doses of capsaicin; Figure 4.7). These responses

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remained unaffected after treatment with either vehicle or sumatriptan (300 μg kg^{-1}, i.v.).

      Finally, it is noteworthy that even with further increasing the dose of sumatriptan (i.e., 1000 μg kg^{-1} min^{-1}, i.v.), the above responses to capsaicin (carotid haemodynamic changes as well as increased plasma CGRP concentrations) did not significantly differ from the vehicle-treated animals (data not shown).
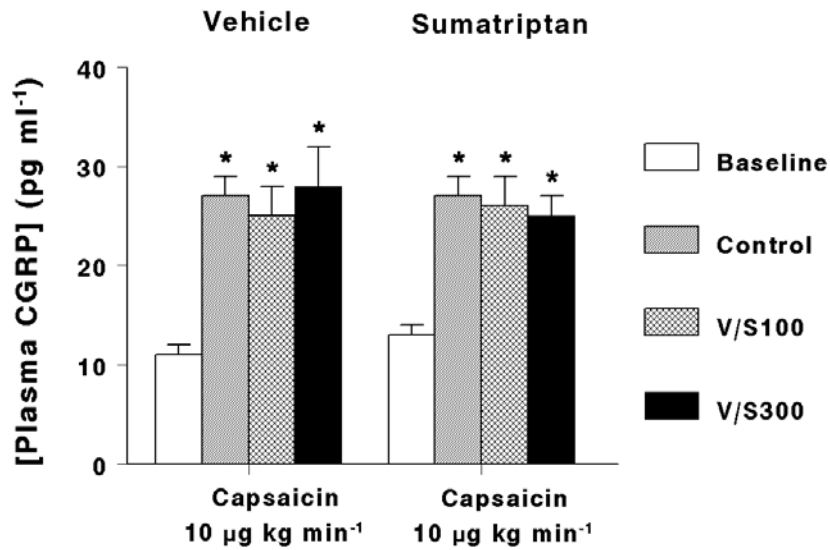
Figure 4.6.   Jugular venous plasma CGRP concentrations measured at baseline and after infusions of capsaicin (10 μg kg^{-1} min^{-1}, i.c.) given in anaesthetised pigs before (Control) and after i.v. administrations of vehicle (V, 5 ml, two times; n=8) or sumatriptan (S100 and S300, 100 and 300 μg kg^{-1}, respectively; n=6).   All values are expressed as mean±s.e.mean. *, P < 0.05 vs. baseline value.
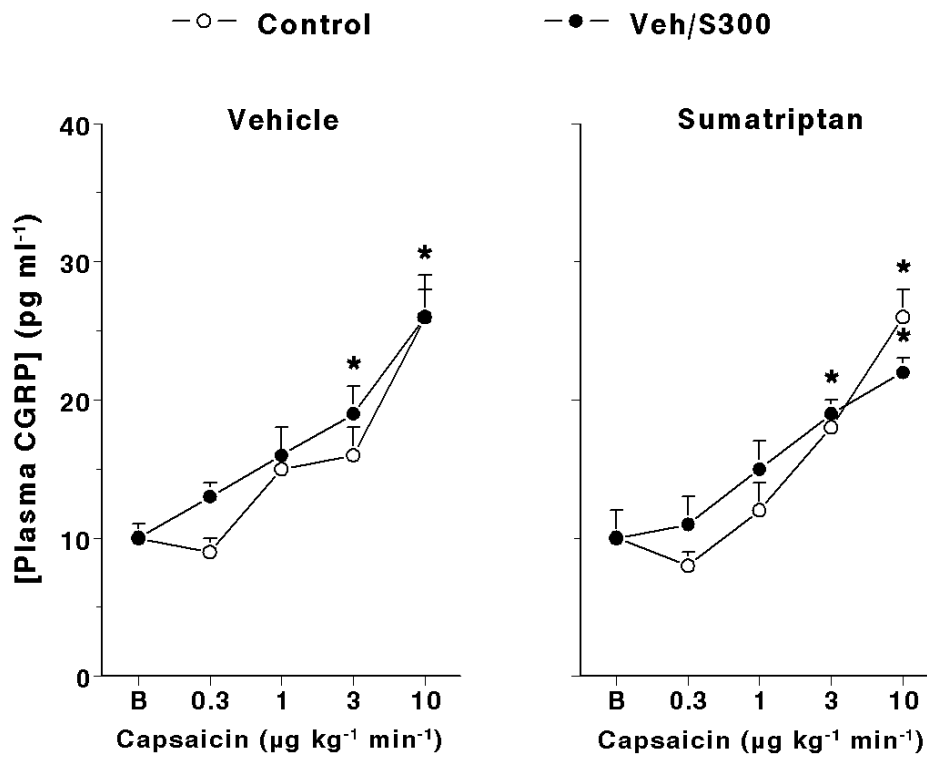
Figure 4.7. Jugular venous plasma CGRP concentrations measured at baseline (B) and after different doses of capsaicin infusion (0.3, 1, 3 and 10 $\mu g\,kg^{-1}\,min^{-1}$, i.c.) given in anaesthetised pigs before (Control) and after i.v. administrations of vehicle (Veh, 5 ml, two times; n=7) or sumatriptan (S300, 300 $\mu g\,kg^{-1}$; n=7). All values are expressed as mean±s.e.mean. *, $P < 0.05$ vs. baseline values.

4.4 Discussion

4.4.1 General

Migraine headache is a neurovascular syndrome in which the neural events seem to involve stimulation of the trigeminal system and an ensuing release of CGRP from perivascular trigeminal nerves (Goadsby *et al.*, 2002b; Edvinsson, 2003).

The introduction of triptans (5-HT$_{1B/1D/1F}$ receptor agonists) in migraine therapy has focussed on the role of serotonin receptors in migraine (Tfelt-Hansen *et al.*, 2000; Villalón *et al.*, 2002). Triptans abort migraine attacks by several mechanisms, including: (i) constriction of dilated cranial blood vessels and carotid arteriovenous anastomoses via the stimulation of 5-HT$_{1B}$ receptors (De Vries *et al.*, 1998; De Vries *et al.*, 1999c); and (ii) inhibition of CGRP release as well as of nociceptive transmission on peripheral and central trigeminal sensory nerves via 5-HT$_{1B/1D}$ receptors (Goadsby *et al.*, 2002b; Tepper *et al.*, 2002). Since trigeminal inhibition of CGRP release may reduce cranial vasodilatation and nociception, the present study set out to investigate the effects of sumatriptan on capsaicin-induced porcine carotid haemodynamic changes and the associated increase on plasma CGRP concentrations. Our results in anaesthetised pigs show that i.c. administration of capsaicin: (i) increased total carotid (including arteriovenous anastomoses and capillary) blood flows and conductances, carotid pulsations as well as jugular venous plasma CGRP concentrations; and (ii) narrowed the A-V SO$_2$ difference. Interestingly, sumatriptan failed to modify capsaicin-induced: (i) carotid haemodynamic changes, except carotid arteriovenous anastomoses; and (ii) increase in plasma CGRP concentrations.

4.4.2 Systemic haemodynamic effects of capsaicin

The systemic haemodynamic effects of capsaicin have been investigated extensively (Alving & Franco-Cereceda, 1993; Kapoor *et al.*, 2003a). Indeed, the significant increases in heart rate and mean arterial blood pressure observed in our study with capsaicin are in accordance with these findings, which are due to a central activation of the sympathetic outflow.

4.4.3   Carotid haemodynamic changes to capsaicin

Several studies have shown that sensory nerves innervating the cerebral vasculature contain substance P and CGRP (Asari *et al.*, 2001; Edvinsson, 2001b). However, capsaicin-induced vasorelaxation of guinea pig isolated basilar artery is mediated by CGRP rather than by substance P (Jansen *et al.*, 1990; O'Shaughnessy *et al.*, 1993). Moreover, BIBN4096BS, a potent CGRP receptor antagonist, abolished capsaicin-induced porcine carotid haemodynamic responses (Kapoor *et al.*, 2003a), a finding which shows the involvement of CGRP in the responses to capsaicin.

Apart from some attenuation of arteriovenous anastomotic changes, the capsaicin-induced responses were not affected by sumatriptan (Figures 4.1-4.4). These results are in keeping with other findings showing that sumatriptan failed to block capsaicin-induced relaxation of guinea pig isolated basilar artery (O'Shaughnessy *et al.*, 1993) as well as carotid vasodilatation induced by trigeminal ganglion stimulation (Spokes & Middlefell, 1995; Lambert & Michalicek, 1996; Raval *et al.*, 1999).

It is noteworthy that the decrease in total carotid conductance by sumatriptan is predominantly due to the decrease in carotid arteriovenous anastomotic conductance, even when all triptans slightly increase the nutrient conductance (Den Boer *et al.*, 1992; De Vries *et al.*, 1996b). Considering this, it is most likely that the above apparent inhibition by sumatriptan on capsaicin-induced responses (Figure 4.3) is due to physiological antagonism (i.e., vasoconstriction by sumatriptan) rather than inhibition of CGRP release, since sumatriptan failed to modify this variable (see Figures 4.6 and 4.7). In contrast, the apparent failure of sumatriptan to attenuate capsaicin-induced increase in carotid conductance may have been due to: (i) massive bursts of CGRP release produced by capsaicin; and/or (ii) an insufficient dose of sumatriptan. However, the latter possibility can be excluded as treatment with an even higher dose of sumatriptan ($1000\ \mu g\ kg^{-1}\ min^{-1}$, i.v.) did not modify capsaicin-induced carotid haemodynamic changes (unpublished data); thus, the involvement of $5\text{-HT}_{1B/1D}$ receptors, if any, seems to be rather limited under the present experimental conditions.

4.4.4   A-V SO$_2$ difference

The dilatation of carotid arteriovenous anastomoses with an associated decrease in the A-V SO$_2$ difference is a characteristic feature observed during migraine headache (Heyck, 1969). Consistent with the above finding, our study shows that capsaicin decreased the A-V SO$_2$ difference, which is presumably due to a CGRP-mediated dilatation of carotid arteriovenous anastomoses (Kapoor *et al.*, 2003b). As expected, sumatriptan decreased the carotid arteriovenous anastomotic conductance in our study (Figure 4.3); however, its failure to normalise the capsaicin-induced A-V SO$_2$ difference may be explained in terms that capsaicin-induced increase in capillary conductance may lead to oxygen saturation in the cranial tissues. Therefore, these tissues cannot extract oxygen further, thereby shunting the oxygenated blood via capillaries rather than via carotid arteriovenous anastomoses.

4.4.5   Plasma levels of CGRP

Several lines of evidence have shown that stimulation of the trigeminal system with electrical or chemical (capsaicin) stimuli releases the endogenously stored CGRP (Buzzi *et al.*, 1991; Goadsby, 1993; O'Shaughnessy *et al.*, 1993; Knight *et al.*, 1999; Eltorp *et al.*, 2000; Limmroth *et al.*, 2001; Kapoor *et al.*, 2003a), which produces cranial vasodilatation (Goadsby & Edvinsson, 1993; Akerman *et al.*, 2003; Kapoor *et al.*, 2003a). Interestingly, triptans have been reported to attenuate CGRP release elicited by both electrical (Buzzi *et al.*, 1991; Goadsby, 1993; Williamson *et al.*, 1997; Knight *et al.*, 1999; Limmroth *et al.*, 2001) and chemical (capsaicin) (Eltorp *et al.*, 2000) stimulation; the latter effect was barely significant and observed with sumatriptan in a concentration (50 μM) that may be considered far beyond the therapeutic range (Eltorp *et al.*, 2000). Indeed, this inhibitory effect on CGRP release in cats has been associated with a blockade of the resulting cranial vasodilatation (Goadsby & Edvinsson, 1993) although, admittedly, this may also reflect a physiological antagonism produced by the triptans-induced vasoconstriction (De Vries *et al.*, 1999a). Considering these findings, it would seem reasonable to suggest that trigeminal (CGRP release) inhibition may be an additional mechanism behind the antimigraine action of triptans.

Notwithstanding, other lines of evidence seem to show just the opposite, namely, that triptans do not modify the cranial vasodilatation produced by either

trigeminal stimulation (Lambert & Michalicek, 1996; Raval *et al.*, 1999) or capsaicin (O'Shaughnessy *et al.*, 1993). Admittedly, in these studies CGRP concentrations were not measured in parallel and, consequently, no categorical conclusion can be drawn regarding the antimigraine action of triptans. Our *in vivo* study sheds further light on this matter by showing that: (i) sumatriptan failed to modify the capsaicin-induced increases in plasma CGRP levels and the associated carotid vasodilatation; and (ii) the carotid arteriovenous anastomotic vasoconstriction to sumatriptan is not associated with a corresponding change in CGRP levels.

Admittedly, there is no clear-cut explanation why sumatriptan seems to attenuate CGRP release upon electrical stimulation but not by capsaicin. It is known that capsaicin activates a subset of small sensory fibres that cover a major proportion of C and some Aδ fibres (Dray, 1992a; Dray, 1992b; Urban & Dray, 1992; Akerman *et al.*, 2003), while low intensity electrical stimulation recruits Aδ fibres alone (Akerman *et al.*, 2003). Thus, one explanation for the differential effect of sumatriptan may be that, compared to electrical stimulation, capsaicin releases high quantities of CGRP that can be potently antagonised by selective vanilloid VR_1 antagonists (Caterina *et al.*, 1997), but not by a presynaptic mechanism involving sumatriptan. Another possibility is that, in contrast to the capsaicin-sensitive C and δ fibres, the δ fibres recruited by electrical stimulation possess 5-HT$_{1B/1D/1F}$ receptors stimulated by sumatriptan (Tfelt-Hansen *et al.*, 2000; Goadsby *et al.*, 2002b). We have indeed shown that the *mRNAs* for 5-HT$_{1B}$ and 5-ht$_{1F}$ receptors are expressed in the porcine trigeminal ganglia (Bhalla *et al.*, 2001; Bhalla *et al.*, 2002). Finally, sumatriptan does not easily penetrate the blood brain barrier (Tfelt-Hansen *et al.*, 2000) and, should CGRP be mainly released from intracerebral sources, it will inhibit such a release only if the blood brain barrier is disrupted.

4.4.6   Possible clinical implications

Finally, the possible clinical implications of our results with sumatriptan within the context of antimigraine therapy must be considered. Therefore, based on our findings, the blockade of the postjunctional effects of CGRP (with BIBN4096BS) (Kapoor *et al.*, 2003a) would seem to be a better therapeutic strategy to prevent neurogenic vasodilatation rather than trigeminal inhibition of CGRP release (via the activation of

prejunctional 5-HT$_{1B/1D}$ receptors by sumatriptan). Moreover, in view of the putative pathophysiological role of arteriovenous anastomotic dilatation in migraine (Heyck, 1969; Saxena, 1995), the constriction of these non-nutrient vessels by sumatriptan in our study may be responsible for the therapeutic action of this drug in migraine.

In conclusion, our results imply that prejunctional 5-HT$_{1B/1D}$ receptors (activated by sumatriptan) do not inhibit capsaicin-induced: (i) vasodilatation of the porcine carotid circulation; and (ii) increase in plasma CGRP concentrations. Therefore, the primary mechanism behind the clinical efficacy of sumatriptan in migraine may be due to vasoconstriction of cranial blood vessels rather than neurogenic inhibition of CGRP release.

CHAPTER 5

Effects of the CGRP receptor antagonist BIBN4096BS on α-CGRP-induced regional haemodynamic changes in anaesthetised rats

5   Effects of the CGRP receptor antagonist BIBN4096BS on α-CGRP-induced regional haemodynamic changes in anaesthetised rats

Abstract: Several studies have suggested that a calcitonin gene-related peptide (CGRP) receptor antagonist may have antimigraine properties, most probably via the inhibition of CGRP-induced cranial vasodilatation. We have previously shown that BIBN4096BS, a potent and selective CGRP receptor antagonist, attenuated the CGRP-induced porcine carotid vasodilatation in a model predictive of antimigraine activity. In order to evaluate the potential safety of BIBN4096BS in migraine therapy, this study was designed to investigate the effects of intravenous (i.v.) BIBN4096BS on α-CGRP-induced systemic and regional haemodynamic changes in anaesthetised rats. In vehicle-pretreated animals, consecutive i.v. infusions of α-CGRP (0.25, 0.5 and 1 $\mu g\,kg^{-1}\,min^{-1}$) dose-dependently decreased mean arterial blood pressure with an accompanying increase in heart rate and systemic vascular conductance whereas cardiac output remained unchanged. α-CGRP also increased the vascular conductance to the heart, brain, gastrointestinal tract, adrenals, skeletal muscle and skin, whilst that to the kidneys, spleen, mesentery/pancreas and liver remained unaltered. The above systemic and regional haemodynamic responses to α-CGRP were clearly attenuated in BIBN4096BS (3000 $\mu g\,kg^{-1}\,min^{-1}$; i.v.)-pretreated animals. These lines of evidence indicate that exogenously administered α-CGRP dilates regional vascular beds via CGRP receptors on the basis of the antagonism produced by BIBN4096BS. Moreover, the fact that BIBN4096BS did not alter baseline haemodynamics suggests that endogenously produced CGRP does not play an important role in regulating the systemic and regional haemodynamics under resting conditions.

5.1   Introduction

Migraine is a neurovascular syndrome thought to be associated with profound dilation of cranial blood vessels and activation of the trigeminovascular system (Saxena & Tfelt-Hansen, 2000; Goadsby *et al.*, 2002b). Several studies have shown that vasoactive neuropeptides (e.g., neuropeptide Y, substance P, calcitonin gene-related peptide; CGRP) may be involved in the aetiology of this disorder (Edvinsson, 2001b; Goadsby *et al.*, 2002b). Interestingly, circulating plasma levels of α-CGRP (a 37-amino acid neuropeptide), but not of other neuropeptides, are significantly elevated during the headache phase of a migraine attack (Ashina *et al.*, 2000; Goadsby *et al.*, 2002b) and these elevated α-CGRP levels are normalised by antimigraine agents, such as sumatriptan, with complete resolution of headache (Goadsby & Edvinsson, 1993).

These findings suggest that CGRP may play a predominant role in migraine pathogenesis, possibly by dilating large cranial blood vessels (Williamson & Hargreaves, 2001; Goadsby *et al.*, 2002b). Therefore, compounds inhibiting either the CGRP release or its effects, particularly cranial vasodilatation, may be efficacious in migraine therapy.   In this context, BIBN4096BS, a potent and selective CGRP receptor antagonist (Doods *et al.*, 2000), completely attenuated carotid vasodilatation by endogenously released (by capsaicin) and exogenously administered CGRP (Kapoor *et al.*, 2003a; Kapoor *et al.*, 2003b) in an experimental animal model predictive of antimigraine activity (Saxena, 1995; De Vries *et al.*, 1999a). Besides its potential efficacy, the therapeutic effectiveness of BIBN4096BS in acute migraine treatment will also depend on its pharmacokinetic properties and potential side effects in humans. With respect to the latter, it has been shown that BIBN4096BS attenuates CGRP-induced dilatation of human isolated coronary arteries (Edvinsson *et al.*, 2002), a vascular bed that is largely affected by the currently used antimigraine agents (Maassen VanDenBrink *et al.*, 1999).

On the basis of the above, the present study set out to analyse in anaesthetised rats, the effects produced by i.v. administration of BIBN4096BS on: (i) baseline systemic haemodynamics (to investigate its the potential cardiovascular side effects); and (ii) the systemic and regional haemodynamic responses to α-CGRP (to ascertain CGRP receptor distribution).

5.2   Materials and methods

5.2.1   General

Experiments were carried out in 13 male Wistar rats (body weight: 356±35 g) obtained from Harlan, Zeist, The Netherlands.   The animals were initially anaesthetised with an intraperitoneal (i.p) injection of sodium pentobarbitone (60 mg kg^{-1}, i.p), and additional i.v. bolus injections (5 mg kg^{-1}, i.v.) were provided every 20-30 min to maintain the anaesthesia. A catheter was placed in the trachea for intermittent positive pressure ventilation with a mixture of oxygen and room air, using a respiratory pump (small animal ventilator, Harvard Apparatus, Natick, MA, USA). The ventilation rate was adjusted (40 strokes min^{-1}) to keep the arterial blood gases within the physiological range. The right common carotid artery was exposed and a catheter connected to a pressure transducer (Combitrans disposable pressure

transducer, Braun, Melsungen, Germany) was guided through the carotid artery into the left ventricle. The presence of the catheter tip in the left ventricle was confirmed by the observation of a sudden switch from an arterial to a ventricular pressure profile. The right femoral artery was catheterised and connected to a pressure transducer (Combitrans disposable pressure transducer, Braun, Melsungen, Germany) for recording the blood pressure, while the left femoral artery was catheterised for the withdrawal of reference blood samples. The heart rate was measured with a tachograph (CRW, Erasmus Medical Centre, Rotterdam, The Netherlands) triggered by electrocardiogram signals. Both blood pressure and heart rate were recorded simultaneously on a polygraph (CRW, Erasmus Medical Centre, Rotterdam, The Netherlands). The right external jugular vein was catheterised for the administration of compounds (α-CGRP and BIBN4096BS or the corresponding volume of vehicle).

5.2.2   Distribution of cardiac output

The distribution of cardiac output was determined with radioactive microspheres (diameter: 15.5 ± 0.1 µm; S.D.), labelled with ^{141}Ce, ^{103}Ru, ^{95}Nb or ^{46}Sc (NEN Dupont, Boston, USA). For each measurement, about 200,000 microspheres, suspended in 0.2 ml of physiological saline and labelled with one of the isotopes, was mixed and injected into the left ventricle over a period of 15 s; the catheter was thoroughly flushed with 0.5 ml of saline. Starting 10 s before each microsphere injection and lasting 70 s, an arterial reference blood sample was drawn from the left femoral artery at a constant rate of 0.5 ml min^{-1}, using a withdrawal pump (Model 55, Harvard apparatus, Natick, USA). At the end of the experiment, the animal was killed using an overdose of sodium pentobarbitone and all tissues were dissected out, weighed and put in vials. The following tissues were studied: skeletal muscle; carcass (consisting of bone with skeletal muscle residue, fat, tail, eyes, and urogenital tract); mesentery/pancreas (for practical reasons, these two tissues were not studied separately); adrenals; lungs; kidneys; skin; heart; liver; brain; gastrointestinal tract and spleen. Lungs were not evaluated further, because the amount of radioactivity in these organs represents the microspheres that bypassed the peripheral vascular beds (via arteriovenous anastomoses), rather than pulmonary blood flow (Baile *et al.*, 1982). The radioactivity in the reference blood samples and in the tissues was

counted for 5 min in a γ-scintillation counter (Packard, Minaxi Auto-Gamma 5000 series), using suitable windows for the discrimination of the different isotopes (^{141}Ce: 120-167   KeV, ^{103}Ru: 450-548   KeV, ^{95}Nb: 706-829   KeV   and ^{46}Sc: 830-965 KeV). All data were processed by a set of specially designed computer programs (Saxena *et al.*, 1980).

The cardiac output was calculated by multiplying the ratio of total and arterial blood sample radioactivity by the withdrawal rate of the arterial reference blood sample (0.5 ml min^{-1}). Accordingly, tissue blood flow was calculated by multiplying the ratio of the tissue and total radioactivity by cardiac output (Saxena *et al.*, 1980). Systemic and regional vascular conductances (i.e., cardiac output and regional blood flow corrected for mean arterial blood pressure) were calculated, multiplied by hundred and expressed as 10^{-2} ml mmHg^{-1} min^{-1}.

5.2.3   Experimental protocol

The experiments were started after a stabilisation period of about 30 min. At this point, the animals were divided into two groups. The first group (n=6) was pre-treated with the vehicle of BIBN4096BS (0.5 ml of acidified distilled water; 0.05 ml min^{-1} for 10 min; i.v.; see Compounds section), while the second group (n=7) was pre-treated with BIBN4096BS (3000 μg kg^{-1}, i.v.; also at a rate of 0.05 ml min^{-1} for 10 min). After a waiting period of 10 min, the baseline values of heart rate, mean arterial blood pressure, cardiac output and its distribution to the various tissues (see above) were determined and both groups received sequential i.v. infusions of α-CGRP (0.25, 0.5 and 1 μg kg^{-1} min^{-1}; each dose for 10 min). After each dose of α-CGRP infusion, the above mentioned haemodynamic variables were reassessed.

5.2.4   Data presentation and statistical analysis

All data are presented as mean±s.e.mean. The statistical analysis was performed using the SPSS package for windows (version 10.0; SPSS Inc., Chicago, IL, USA). The significance of changes within one group (vehicle or BIBN4096BS) was analysed with repeated-measures ANOVA, followed by Greenhouse-Geisser correction for serial autocorrelation (Ludbrook, 1994) and Bonferroni correction for multiple comparisons (Overall & Doyle, 1996). The significance of the between-group changes (vehicle vs. BIBN4096BS treatments) was first analysed with repeated-measures ANOVA, including baseline measurements as a covariate (Overall & Doyle,

1994).   If the two groups differed significantly, pairwise comparisons of the corresponding values in the vehicle- and BIBN4096BS-treated groups were performed using univariate analysis (Overall & Atlas, 1999), followed by Bonferroni correction. Statistical significance was accepted at $P<0.05$ (two-tailed).

5.2.5   Ethical approval

The Ethics Committee of the Erasmus MC, Rotterdam, The Netherlands, dealing with the use of animals in scientific experiments, approved the protocols for the present investigation.

5.2.6   Compounds

The following compounds were used: sodium pentobarbitone (Sanofi Sante b.v., Maasluis, The Netherlands), heparin sodium (to prevent clotting of blood in the catheters; Leo Pharmaceutical Products, Weesp, The Netherlands), α-CGRP and BIBN4096BS (both gifts from Dr. H. Doods, Boehringer Ingelheim Pharma KG, Biberach, Germany).

α-CGRP was dissolved in distilled water, while BIBN4096BS was initially dissolved in 0.5 ml of 1N HCl, then diluted with 4 ml of distilled water, and then adjusted to pH 6.5 by 1N NaOH.

5.3   Results

5.3.1   Baseline values of systemic and regional haemodynamic variables

Baseline values of systemic haemodynamic variables in the 13 anaesthetised rats used in this investigation were: heart rate, 266 ± 10 beats min^{-1}; mean arterial blood pressure, 108 ± 3 mmHg; cardiac output, 68 ± 4 ml min^{-1} and systemic vascular conductance, 64 ± 4 10^{-2} ml min^{-1} $mmHg^{-1}$.   Baseline values of regional vascular conductances (10^{-2} ml min^{-1} $mmHg^{-1}$/100g tissue) were: brain, 32 ± 4; heart, 409 ± 58; liver, 35 ± 3; gastrointestinal tract, 124 ± 24; mesentery/pancreas, 32 ± 4; adrenals, 257 ± 52; kidneys, 417 ± 38; spleen, 92 ± 24; skeletal muscle, 5 ± 0.4; and skin, 5 ± 1. These values were similar to those reported earlier from our laboratories (Schuijt *et al.*, 1999).

The baseline values in the animals pre-treated with vehicle (n=6) and BIBN4096BS (n=7) did not differ significantly: heart rate (275 ± 19 vs. 259 ± 8 beats min^{-1}); mean arterial blood pressure (108 ± 3 vs. 108 ± 5 mmHg); cardiac output

(68±6 vs. 69±6 ml min^{-1}); systemic vascular conductance (63±6 vs. 65±7 10^{-2} ml min^{-1} mmHg^{-1}) and regional vascular conductances (10^{-2} ml min^{-1} mmHg^{-1}/100g tissue) in brain (32±5 vs. 33±6), heart (393±101 vs. 423±72), liver (33±6 vs. 36±2), gastrointestinal tract (103±16 vs. 141±43), mesentery/pancreas (33±7 vs. 32±4), adrenals (282±96 vs. 235±58), kidneys (445±56 vs. 394±54), spleen (100±50 vs. 85±16), skeletal muscle (5±0.5 vs. 4±0.5) and skin (6±1 vs. 4±0.7).

5.3.2   Systemic haemodynamic responses to α-CGRP

The absolute changes in systemic haemodynamics following consecutive i.v. infusions of α-CGRP (0.25, 0.5 and 1 μg kg^{-1} min^{-1}; i.v.) in the animals pre-treated with BIBN4096BS (3 mg kg^{-1}, i.v.) or the corresponding volume of vehicle (acidified distilled water, 0.05 ml; i.v) are shown in Figure 5.1.   The infusions of α-CGRP dose-dependently decreased mean arterial blood pressure (maximum percent change from baseline: 55±5) with an increase in systemic vascular conductance (maximum percent change from baseline: 56±21) and heart rate (maximum percent change from baseline: 17±3).   BIBN4096BS pre-treatment produced an attenuation of the systemic haemodynamic responses produced by α-CGRP.   Under these conditions, the highest dose of α-CGRP still elicited small, though significant, decreases (maximum percent changes from baseline) in: (i) mean arterial blood pressure (28±4 vs. 51±7 in vehicle pre-treated animals); and (ii) systemic vascular conductance (16±6 vs. 27±8 in vehicle pre-treated animals).

5.3.3   Regional haemodynamic responses to α-CGRP

Figure 5.2 depicts absolute changes in regional vascular conductance following consecutive i.v. infusions of α-CGRP (0.25, 0.5 and 1 μg kg^{-1} min^{-1}; i.v.) in the animals pre-treated with BIBN4096BS (3 mg kg^{-1}, i.v.) or the corresponding volume of vehicle.   α-CGRP increased the vascular conductances (maximal percent change from baseline) to brain (124±45), gastrointestinal tract (80±35), heart (74±31), adrenals (87±37), muscle (79±27) and skin (154±37), whilst that to the mesentery/pancreas, liver, spleen and kidneys remained unchanged.   BIBN4096BS attenuated the above regional haemodynamic changes induced by α-CGRP.   The vascular conductance to the kidneys decreased significantly in vehicle-pretreated

animals following the highest dose of α-CGRP (1 μg kg^{-1} min^{-1}; i.v.) infusion, but this was not the case in the animals pre-treated with BIBN4096BS.

Human α-CGRP (μg kg^{-1} min^{-1}, i.v.)
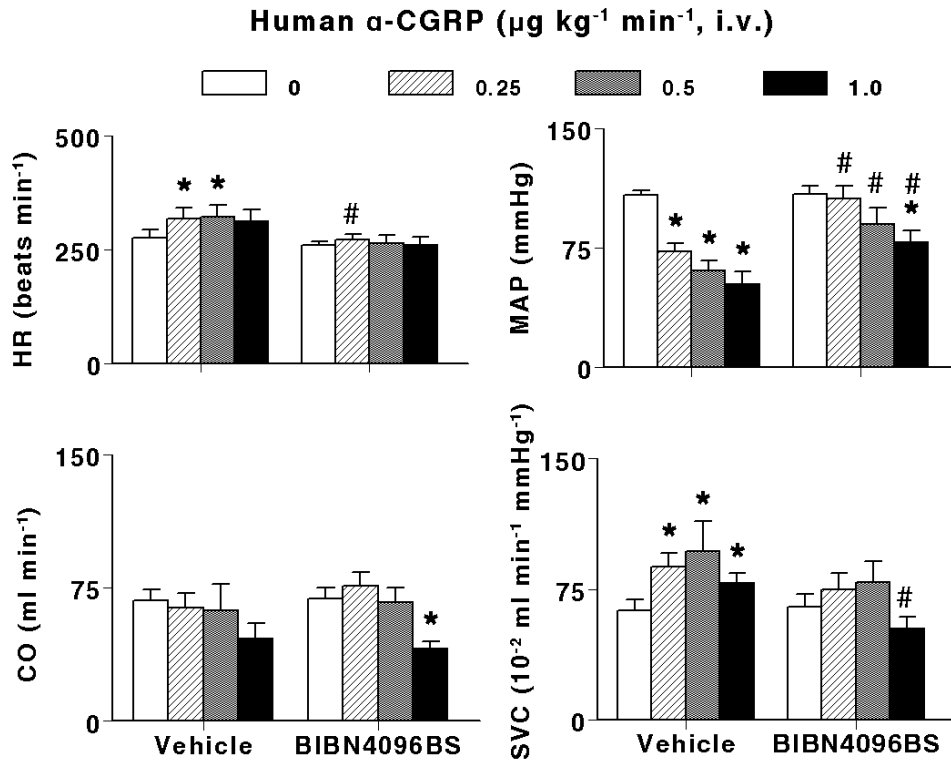
Figure 5.1. Absolute values of heart rate (HR), mean arterial blood pressure (MAP), cardiac output (CO) and systemic vascular conductance (SVC) before (α-CGRP, 0 μg kg^{-1} min^{-1}; baseline) and following infusions of α-CGRP (0.25, 0.5 and 1 μg kg^{-1} min^{-1}; i.v.) in anaesthetised rats pre-treated with BIBN4096BS (3 mg kg^{-1}, i.v.; n=7) or the corresponding volume of vehicle (0.05 ml; i.v.; n=6). All values are expressed as mean±s.e.mean. *, P < 0.05 compared to baseline values; $^{#}$, P<0.05 compared to the corresponding dose in vehicle-pretreated animals.
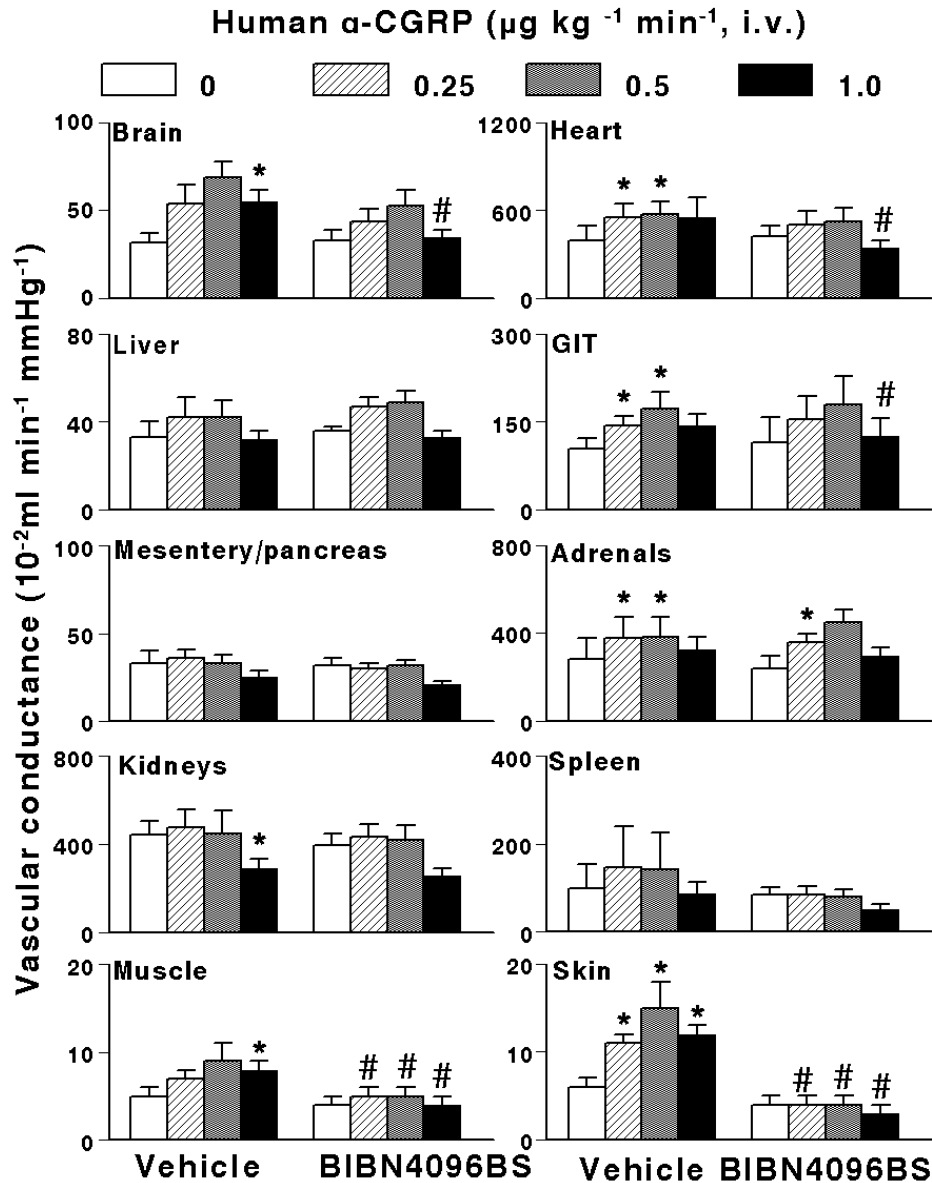
Figure 5.2. Absolute values of regional vascular conductances before (α-CGRP, 0 μg kg^{-1} min^{-1}; baseline) and following infusions of α-CGRP (0.25, 0.5 and 1 μg kg^{-1} min^{-1}; i.v.) in anaesthetised rats pre-treated with BIBN4096BS (3 mg kg^{-1}, i.v.; n=7) or the corresponding volume of vehicle (0.05 ml; i.v.; n=6). GIT: gastrointestinal tract.   All values are expressed as mean±s.e.mean. *, P < 0.05 compared to baseline values; #, P<0.05 compared to the corresponding dose in vehicle-pretreated animals.

5.4 Discussion

5.4.1 General

CGRP receptors are widely distributed throughout the body and are predominantly expressed in the nervous system including perivascular nerves (Okimura *et al.*, 1987; Sternini *et al.*, 1992). Therefore, CGRP may play an important role in regulating peripheral vascular tone and in controlling blood flow to various organs (Poyner *et al.*, 2002). Several lines of evidence suggest that an increase in the release of CGRP is a potential causative factor in some pathological conditions including migraine (Wimalawansa, 1996). Hence, the advent of BIBN4096BS may represent an important headway in treating migraine.

Apart from the implications discussed below, our study in anaesthetised rats shows that BIBN4096BS: (i) had no effect on baseline systemic and regional haemodynamics, confirming its cardiovascular safety; and (ii) antagonised α-CGRP-induced changes in systemic and regional haemodynamics, demonstrating the wide distribution of CGRP receptors.

5.4.2 Systemic haemodynamic changes to α-CGRP

The potential role of CGRP in regulating the systemic haemodynamics has been studied extensively in several species, including humans (Franco-Cereceda *et al.*, 1987; Ventura *et al.*, 2000; Rasmussen *et al.*, 2001). Accordingly, CGRP decreases systemic blood pressure through its potent vasodilator effect on the peripheral vasculature (van Rossum *et al.*, 1997). The fact that BIBN4096BS blocked the hypotensive responses to α-CGRP (Figure 5.1) confirms a CGRP receptor-mediated response. Interestingly, the hypotensive response to the highest dose of α-CGRP ($1 \ \mu g \ kg^{-1} \ min^{-1}$; i.v.) was just partly blocked by BIBN4096BS; this may be attributed, at least in part, to the lower affinity of BIBN4096BS for rat CGRP receptors (Doods *et al.*, 2000). Consistent with our findings, it has been reported that BIBN4096BS is more potent than $CGRP_{8-37}$ at rat CGRP receptors (Poyner *et al.*, 2002). However, a non-specific vasodilatation to CGRP via the production of nitric oxide cannot be categorically excluded in our experiments (Akerman *et al.*, 2002; de Hoon *et al.*, 2003).

On the other hand, with respect to the tachycardic responses to α-CGRP observed in the present study, it is worthy of note that cardiac CGRP receptors are more abundant in the sinoatrial node and atria than in the ventricles (Du *et al.*, 1994; Bell & McDermott, 1996; Wimalawansa, 1996; Saetrum Opgaard *et al.*, 2000). Most importantly, other lines of evidence have shown that this response is resistant to β-adrenoceptor antagonists, suggesting a direct chronotropic action of α-CGRP in the rat heart (Marshall *et al.*, 1986). However, a baroreceptor reflex mechanism (which cannot be entirely ruled out in our experimental set-up) may also be involved.

Noteworthily, CGRP is a potent inotropic agent in rabbits, pigs as well as humans (Van Gelderen *et al.*, 1995; Bell & McDermott, 1996), but not in rats (Ishikawa *et al.*, 1987; Bratveit *et al.*, 1991). This may explain the lack of effect of α-CGRP on cardiac output in our study (Figure 5.1).

5.4.3 Regional haemodynamic changes by α-CGRP

The regional haemodynamic responses to α-CGRP observed in our study clearly demonstrate the vasodilator properties of CGRP in different vascular beds. Furthermore, the CGRP-induced increases in vascular conductances in the heart, brain, gastrointestinal tract, adrenals, skin and skeletal muscle were attenuated by BIBN4096BS (Figure 5.2), indicating that these responses are mediated via CGRP receptors. However, tissues such as liver, spleen, mesentery/pancreas and kidneys did not apparently show any changes to CGRP. Several possible explanations for this lack of effect of CGRP may include, amongst others: (i) tissue-dependent factors such as the density of CGRP receptors and coupling efficiency; and/or (ii) a blunting effect via the activation of compensatory pressor mechanisms triggered by the prominent reduction in systemic blood pressure (DiPette *et al.*, 1989; Gardiner *et al.*, 1990).

5.4.4 Clinical implications of CGRP antagonism by BIBN4096BS

Lastly, we would like to consider the possible clinical implications of BIBN4096BS safety in antimigraine therapy. BIBN4096BS, which is effective in antagonising CGRP-induced responses in both *in vivo* and *in vitro* studies (Edvinsson *et al.*, 2002; Kapoor *et al.*, 2002; Moreno *et al.*, 2002; Verheggen *et al.*, 2002; Kapoor *et al.*, 2003b), has been shown effective in a phase II clinical trial for acute antimigraine therapy (Edvinsson, 2003; Olesen *et al.*, 2003a). In this context, our study shows that

BIBN4096BS did not compromise the blood flow in a number of tissues, even in a dose that may be considered relatively higher than that used in the clinic. Thus, BIBN4096BS does not show any unwanted cardiovascular effects in our experiments.

In conclusion, the present investigation demonstrates that: (i) exogenously administered α-CGRP dilates several regional vascular beds in a dose-dependent manner; and (ii) endogenous CGRP does not play an important role in regulating systemic and regional haemodynamics.

CHAPTER 6

Discussion

6 DISCUSSION

6.1 General

Though the precise mechanism behind migraine pathogenesis is still far from clear, some lines of evidence suggest an involvement of extracranial arterial vasodilatation, extracranial neurogenic inflammation and/or a decreased inhibition of central pain transmission (Spierings, 2003). It is undeniable that the cranial vasoconstrictor activity of the triptans, mediated by 5-HT_{1B} receptors, is associated with antimigraine efficacy (De Vries *et al.*, 1999a; De Vries *et al.*, 1999b). Unfortunately, the 5-HT_{1B} receptor, being not exclusively confined to cranial blood vessels, is most likely also responsible for the moderate hypertension and coronary constriction noticed with these drugs (Villalón *et al.*, 2002).

In an attempt to avoid coronary vasoconstriction, some new avenues are being explored for the development of novel antimigraine agents, including the antagonism of receptors for CGRP and the inhibition of trigeminal CGRP release. Hence, the discovery and development of an antimigraine agent capable of inhibiting trigeminal CGRP release (and the associated cranial vasodilatation) or of antagonising selectively cranial CGRP receptors without producing vasoconstriction would be a tremendous achievement in the clinical treatment of migraine (Goadsby *et al.*, 2002b; Villalón *et al.*, 2002).

Several findings support the potential role of CGRP in migraine pathogenesis, including its ability to dilate cranial blood vessels and to stimulate central nociception transmission (Edvinsson, 2001b; Olesen & Lassen, 2001; Goadsby *et al.*, 2002b). With this in mind, the CGRP receptor antagonist, BIBN4096BS, which has the highest selectivity for human CGRP receptors, was developed as an agent with potential therapeutic usefulness in the acute treatment of migraine. Indeed, preclinical studies demonstrated its efficacy as a potent and selective CGRP receptor antagonist (Doods *et al.*, 2000; Olesen *et al.*, 2003a).

In view that vasoconstriction of porcine carotid arteriovenous anastomoses is an experimental model highly predictive of antimigraine activity (Saxena, 1995; De Vries *et al.*, 1999a), the present thesis was, in principle, conducted to explore the role of CGRP receptors in producing vasodilatation in the (cranial) carotid circulation, which is one of the main features of migraine headache pathophysiology (Saxena, 1995).

Therefore, we investigated the effects of BIBN4096BS (Doods *et al.*, 2000) on both the capsaicin (which releases endogenously stored CGRP- and α-CGRP-induced porcine carotid haemodynamic changes. In addition, we explored the effects of sumatriptan on capsaicin-induced trigeminal CGRP release (see below).

6.2 Capsaicin-induced carotid haemodynamic responses and CGRP release

Several studies have shown that stimulation of trigeminal ganglia/nerve fibres, which innervate cranial blood vessels, release endogenous CGRP (Asari *et al.*, 2001). It is known that capsaicin, a pungent substance from red chilli pepper, can release several neuropeptides, including: (i) CGRP; (ii) substance P; (iii) neuropeptide Y; (iv) neurokinin A; and (v) catecholamines (Alving *et al.*, 1991). Therefore, we administered intracarotid infusions of capsaicin in anaesthetised pigs to stimulate the release of endogenous CGRP.

Indeed, as described in Chapter 2, capsaicin-induced increase in plasma CGRP concentration are in complete agreement with previously published studies (Alving *et al.*, 1991; Alving & Franco-Cereceda, 1993). Moreover, the increase in plasma CGRP concentrations in our study is clearly associated with the vasodilatation of carotid blood vessels including arteriovenous anastomoses. The use of phenylephrine in our experiments is necessitated by the fact that the carotid arterioles and arteriovenous anastomoses are in a dilated state under pentobarbital anaesthesia (Den Boer *et al.*, 1993); therefore, to study the effects of vasodilator agents (e.g., capsaicin-induced CGRP release) one has to constrict them first. Thus, phenylephrine was infused to decrease the total carotid conductance exclusively by constricting carotid arteriovenous anastomoses (Willems *et al.*, 1999). Moreover, capsaicin was infused in increasing doses to construct dose-response curves and to avoid desensitisation of sensory nerves to capsaicin (Szallasi, 2002).

6.3 Effects of BIBN4096BS on capsaicin-induced carotid haemodynamic responses

As described in Chapter 2, capsaicin-induced carotid haemodynamic responses were dose-dependently attenuated by BIBN4096BS; these findings clearly suggest that the above responses are mediated via CGRP receptors. Interestingly, not only did

BIBN4096BS fail to block capsaicin-induced plasma CGRP release, but also there was a modest enhancement of CGRP release. It is known that presynaptic CGRP receptors are likely to be involved in neuronal CGRP uptake into perivascular and capsaicin-sensitive neurones (Sams-Nielsen *et al.*, 2001). Our findings clearly show that following the blockade of presynaptic 'inhibitory' CGRP autoreceptors with BIBN4096BS, a further increase in the capsaicin-induced CGRP release was observed. This effect is similar to the modulation of sympathetic neurotransmission by presynaptic α_2-adrenoceptors (Langer, 1980). Therefore, these results imply that BIBN4096BS behaves as a potent antagonist of capsaicin-induced carotid haemodynamic changes that are mediated via the release of CGRP.

6.4 Effects of sumatriptan on capsaicin-induced carotid haemodynamic responses

Furthermore, we investigated the effects of sumatriptan on capsaicin-induced carotid haemodynamics and on CGRP release (see Chapter 4). Several studies have reported that triptans inhibit trigeminal CGRP release via the activation of presynaptic 5-HT$_{1D}$ receptors (Tfelt-Hansen *et al.*, 2000; Goadsby *et al.*, 2002b). However, this finding was observed following electrical stimulation of the trigeminal system (Buzzi *et al.*, 1991; Moskowitz & Buzzi, 1991; Goadsby, 1993; Goadsby & Edvinsson, 1993; Knight *et al.*, 1999), but not by chemical (capsaicin) (O'Shaughnessy *et al.*, 1993) stimulation. To the best of our knowledge, our study (Chapter 5) seems to be the first to show *in vivo* the failure of sumatriptan to inhibit capsaicin-induced carotid vasodilatation (except a small reduction in carotid conductance and arteriovenous anastomoses) and CGRP release. These findings are in complete agreement with *in vitro* studies (O'Shaughnessy *et al.*, 1993; Zimmermann *et al.*, 2003). Therefore, our study implies that the primary mechanism behind the clinical efficacy of sumatriptan in migraine may be vasoconstriction of cranial blood vessels rather than trigeminal inhibition of CGRP release.

6.5 Effects of BIBN4096BS on α-CGRP-induced carotid haemodynamic responses

It has previously been shown that CGRP is a potent vasodilator in several vascular beds, including the (cranial) carotid vasculature (Wimalawansa, 1996; Wimalawansa,

2001). Noteworthily, administration of α-CGRP in migraineurs causes a migraine-like headache (Lassen *et al.*, 2002). Therefore, we mimicked the above pathophysiological feature of migraine in our porcine model by infusing α-CGRP and investigated the effects of BIBN4096BS on α-CGRP-induced carotid haemodynamic responses.

Our results (Chapter 3) reconfirm and extend previous findings observed in anaesthetised pigs (Van Gelderen *et al.*, 1995) and show that: (i) the carotid circulation is markedly dilated in response to α-CGRP infusions; and (ii) BIBN4096BS behaves as a "silent" antagonist of the carotid vasodilator responses to α-CGRP. On this basis, the involvement of CGRP receptors is clearly established, as previously demonstrated in isolated cranial blood vessels (Edvinsson *et al.*, 2002; Moreno *et al.*, 2002). Significantly, the capability of BIBN4096BS to elicit a complete blockade of the carotid vasodilator responses to α-CGRP in our study makes the involvement of other receptors highly unlikely.

Considering the above findings in anaesthetised pigs, we finally decided to evaluate in anaesthetised rats (Chapter 4) the potential cardiovascular safety of i.v. BIBN4096BS on: (i) systemic and regional haemodynamics under resting conditions, in order to ascertain the potential role of CGRP receptors in regulating these variables; and (ii) α-CGRP-induced systemic and regional haemodynamic changes in order to analyse the distribution of CGRP receptors in the cardiovascular system.

The fact that BIBN4096BS antagonised the increases in regional vascular conductances to the different tissues indicates that CGRP receptors are widely distributed in the cardiovascular system (Chapter 4). These findings are similar to previously reported results in conscious dogs and anaesthetised rats (Shen *et al.*, 2001), although, admittedly, the peptide antagonist CGRP$_{(8-37)}$ (rather than BIBN4096BS) was used; since this antagonist, unlike BIBN4096BS, possesses partial agonist properties on CGRP receptors (Wimalawansa, 1996), no categorical conclusion can be drawn regarding the distribution of CGRP receptors in the above study.

Relevantly, *in vitro* findings have shown that BIBN4096BS possesses a higher affinity for human and marmoset CGRP receptors than for rat CGRP receptors. Our study carried out in anaesthetised rats is in complete agreement with these

findings as the α-CGRP-induced vasodepressor responses were just partly blocked by BIBN4096BS.

6.6 Role of endogenous CGRP in regulating basal vascular tone

Several lines of evidence have shown that endogenous CGRP might be actively released from primary afferent nerves that innervate the cardiovascular system; these are activated in response to appropriate stimuli such as heating, ischaemia, pH changes, etc (Bell & McDermott, 1996). Therefore, it is reasonable to assume that endogenous CGRP might participate in the regulation of basal vascular tone. Interestingly, our studies in anaesthetised pigs and rats (Chapters 3 and 5, respectively) show that the baseline systemic and regional haemodynamics remained unaltered after i.v. administration of BIBN4096BS. These findings suggest that under normal resting conditions, endogenous CGRP does not play a significant role in the regulation of vascular tone. Nevertheless, in anaesthetised pigs, the highest dose of BIBN4096BS (1000 μg kg^{-1}) moderately decreased vascular conductance in the lungs, kidneys, spleen and adrenals (Chapter 3). These changes to BIBN4096BS may not be due to blockade of CGRP receptors, which was clearly evident with lower doses of BIBN4096BS (100 and 300 μg kg^{-1}).

As described in Chapter 1, the plasma concentrations of CGRP are quite low (Wimalawansa, 1996) and this may explain the lack of effects of BIBN4096BS on systemic and regional haemodynamics with BIBN4096BS; alternatively, it could be proposed that CGRP does not tonically regulate the (cardio) vascular tone. Moreover, it has been reported that the potent vasodilatory responses to endogenous CGRP may depend upon the innervation by nerve fibres containing high concentrations of CGRP (Bell & McDermott, 1996). For example, in rats, the superior mesenteric artery exhibits a dense network of CGRP-immunoreactive nerve fibres compared with the femoral artery (Bell & McDermott, 1996); therefore, the superior mesenteric artery may be more responsive to endogenous CGRP than the femoral artery (Bell & McDermott, 1996). However, in *in vivo* situations, the endogenous feedback mechanisms regulate the synthesis and release of CGRP (Bell & McDermott, 1996); this may be an additional possible explanation for the lack of effect of BIBN4096BS in our studies.

In contrast to the above, the role of endogenous CGRP in pathological conditions, such as myocardial infarction, cerebral ischaemia, endotoxic shock and preconditioning (induced by brief ischaemia/reperfusion) has been well documented (Bell & McDermott, 1996; Gangula *et al.*, 2002; Li & Peng, 2002; Yallampalli *et al.*, 2002). Moreover, in hyperdynamic conditions, such as in pregnancy, endogenous CGRP maintains normal fetoplacental development, fetal survival and vascular adaptations (Gangula *et al.*, 2002).

6.7 Pre- and post-junctional CGRP modulation: implications for migraine treatment

The hypothesis underlying the development of antimigraine compounds is based on the involvement of vascular or neural components in migraine (Fusco *et al.*, 2003). It is well known that migraine pathogenesis involves the activation of trigeminal nerves, which may release CGRP that, in turn, promotes neurogenic inflammation and cranial vasodilatation (Goadsby *et al.*, 2002b; Villalón *et al.*, 2002). Moreover, conventional antimigraine compounds, such as triptans, abort migraine attacks by several mechanisms, including: (i) constriction of dilated cranial blood vessels and carotid arteriovenous anastomoses via the stimulation of $5-HT_{1B}$ receptors (De Vries *et al.*, 1999b; Saxena & Tfelt-Hansen, 2000; Tfelt-Hansen *et al.*, 2000; Goadsby *et al.*, 2002b; Villalón *et al.*, 2002); and (ii) inhibition of CGRP release as well as of nociceptive transmission on peripheral and central trigeminal sensory nerves via $5-HT_{1B/1D}$ receptors (Bigal *et al.*, 2002; Goadsby *et al.*, 2002b; Tepper *et al.*, 2002).

Several studies have reported that inhibition of trigeminal CGRP release may underlie the therapeutic efficacy of triptans (Bigal *et al.*, 2002; Goadsby *et al.*, 2002b). Indeed, findings in animals have shown that triptans inhibit trigeminal CGRP release; this is further strengthened by clinical data showing that sumatriptan normalised the elevated CGRP levels with alleviation of migraine headache (Goadsby & Edvinsson, 1993; Knight *et al.*, 1999; Goadsby *et al.*, 2002b). On the other hand, it has been shown that compounds that inhibit neurogenic inflammation (e.g. NK_1 receptor antagonists) and the trigemino-vascular system ($5-HT_{1D}$ receptor agonist; PNU142633) are ineffective in acute migraine treatment (McCall, 1999; Williamson & Hargreaves, 2001). Therefore, it is not clear whether the inhibition of trigeminal CGRP release is an important mechanism behind the therapeutic efficacy of

antimigraine agents. The above effect of triptans (inhibition of trigeminal CGRP release) may be secondary to the alleviation of headache produced by cranial vasoconstriction. Accordingly, it is tempting to suggest that vasoconstriction of cranial blood vessels, including arteriovenous anastomoses, is the most important effect of acutely-acting antimigraine drugs. This suggestion gains weight when considering that: (i) sumatriptan poorly penetrates the central nervous system; (ii) the $5-HT_{1B/1D}$ receptor agonists, alniditan and IS159, which have little affinity for $5-ht_{1F}$ receptors, are at least as effective as sumatriptan in aborting acute migraine attacks (Goldstein *et al.*, 1996; Chaveau & Delaage, 1997; Dingemanse *et al.*, 1999); and (iii) BIBN4096BS is reported to be effective in migraine based on its antagonism of vascular CGRP receptors (Edvinsson, 2003; Olesen *et al.*, 2003a) and on its failure to block capsaicin-induced CGRP release (Chapter 2).

Finally, our experimental findings support the contention that the therapeutic action of antimigraine compounds is mainly due to cranial vasoconstriction rather than inhibition of trigeminal CGRP release. It would be interesting to investigate whether BIBN4096BS (like triptans) is capable of: (i) inhibiting CGRP release following electrical stimulation of the trigeminal system; and (ii) normalising the elevated plasma CGRP levels in migraine patients.

6.8   Implications for future antimigraine therapy

Based on epidemiology data and other lines of evidence (Mathew, 2001), it seems that people suffering from migraine headache worldwide are not adequately treated and there remains a significant unmet need in migraine care. The real challenge to be faced in near future in migraine treatment is to: (i) diagnose migraine early; and (ii) deliver migraine-specific therapies (Brandes, 2002). Regarding the latter, a crucial improvement should be aimed to avoid potent side effects such as coronary vasoconstriction (Villalón *et al.*, 2002). In this respect, the following possible avenues are being explored: (i) $5-HT_{1D}$ receptor agonists; (ii) $5-ht_{1F}$ receptor agonists; (iii) $5-HT_7$ receptor antagonists; (iv) antagonists at CGRP and substance P receptors; (v) agonists at specific α-adrenoreceptor subtypes; (vi) selective adenosine A_1 receptor agonists; (vii) NO synthesis inhibitors; and (viii) other possible avenues.

(I)   5-HT$_{1D}$ RECEPTOR AGONISTS

A series of isochroman-6-carboxamide derivatives, including PNU-109291, have been described as highly selective 5-HT$_{1D}$ receptor agonists (pK$_i$: 5.2 and 9.0 at 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor, respectively) (Ennis *et al.*, 1998). PNU-109291 is devoid of carotid vasoconstrictor effects in the anaesthetised cat, but potently inhibits dural plasma extravasation in the guinea pig (Ennis *et al.*, 1998). Moreover, these 5-HT$_{1D}$ receptor agonists do not produce vasoconstriction in *in vivo* (canine external and internal carotid beds) (Centurión *et al.*, 2001) or *in vitro* (cerebral arteries) (Bouchelet *et al.*, 2000) preparations. More recently, it has been shown that PNU-142633 (congener) is ineffective in patients with migraine (Gómez-Mancilla *et al.*, 2001). Clearly, inhibition of dural plasma extravasation by itself is not predictive of antimigraine activity.

5-HT$_{1D}$ receptors are found in the trigeminal sensory nerves and their ability to shut down firing is dependent on both drug efficacy and firing frequency; hence, the apparent failure of PNU-142633F to relieve migraine could be due to an insufficient efficacy at 5-HT$_{1D}$ receptors (Williamson & Hargreaves, 2001). This strategy may still represent a viable option for migraine therapy, but the compound should lack activity at 5-HT$_{1B}$ receptors and should have an efficacy similar to sumatriptan at 5-HT$_{1D}$ receptors (Villalón *et al.*, 2002).

(II)   5-HT$_{1F}$ RECEPTOR AGONISTS

Two potent 5-ht$_{1F}$ receptor agonists, LY344864 (pK$_i$: 6.3, 6.2 and 8.2 at 5-HT$_{1B}$, 5-HT$_{1D}$ and 5-ht$_{1F}$ receptor, respectively; (Johnson *et al.*, 1997; Phebus *et al.*, 1997) and LY334370 (pK$_i$ values: 6.9, 6.9 and 8.8 at 5-HT$_{1B}$, 5-HT$_{1D}$ and 5-ht$_{1F}$ receptor, respectively (Johnson *et al.*, 1997; Phebus *et al.*, 1997) have been described. Both compounds potently inhibit dural plasma protein extravasation (Johnson *et al.*, 1997; Phebus *et al.*, 1997), but are devoid of vasoconstrictor activity (Bouchelet *et al.*, 2000). Together with the fact that SB224289, which displays little affinity at the 5-ht$_{1F}$ receptor (Hagan *et al.*, 1997), completely antagonises sumatriptan-induced external carotid vasoconstrictor effects (De Vries *et al.*, 1998; Saxena *et al.*, 1998), it is evident that the 5-ht$_{1F}$ receptor is not involved in the vasoconstrictor effects of sumatriptan and the second-generation triptans. It is therefore implied that, if LY334370 turns out to be effective in migraine at doses devoid of 5-HT$_{1B/1D}$ receptor interaction, the

mechanism of action will not be via cranial vasoconstriction. In fact, it has recently been reported that LY334370 is clinically effective to abort a migraine attacks (Goldstein *et al.*, 2001b). However, it has to be emphasised that LY3334370 displayed antimigraine activity at doses that may interact with extracranial vasoconstrictor 5-HT$_{1B}$ receptors (Goldstein *et al.*, 2001b). In the absence of the importance of dural plasma protein extravasation (see above), further experiments will be needed to explain the efficacy of LY334370.

(III)   5-HT$_7$ RECEPTOR ANTAGONISTS

Methysergide and lisuride, prophylactic antimigraine drugs, have high affinity for 5-HT$_7$ receptors (Hoyer *et al.*, 1994). In addition, it has been shown that 5-HT$_7$ receptors mediate vasodilator responses in several vascular tissues (Eglen *et al.*, 1997) including the canine external carotid bed (Villalón *et al.*, 1997). Thus, it would be expected that selective antagonists at 5-HT$_7$ receptors might have antimigraine properties, although this remains to be determined.

(IV)   ANTAGONISTS AT CGRP AND SUBSTANCE P RECEPTORS

Electrical stimulation of the trigeminal ganglion produces release of potent vasodilator peptides such as substance P and CGRP (Goadsby, 1993; Goadsby *et al.*, 2002b). Further evidence suggests that during an attack of migraine an increase in plasma levels of CGRP is observed (Ashina *et al.*, 2000). The release of CGRP is blocked by dihydroergotamine and sumatriptan (Goadsby, 1993; Goadsby *et al.*, 2002b), indicating that the blockade of this mechanism could be another strategy to develop antimigraine drugs. Recently, it has been shown that the CGRP antagonist, BIBN4096BS, potently and dose-dependently inhibited the increases in facial blood flow induced by electrical stimulation of the trigeminal ganglion (Doods *et al.*, 2000). These findings, in conjunction with the ability of BIBN4096BS to antagonise CGRP-induced vasorelaxation of the human temporal artery (Verheggen *et al.*, 2002) and porcine carotid arteriovenous anastomoses (Kapoor *et al.*, 2003a; Kapoor *et al.*, 2003b) strongly suggest that blockade of vascular CGRP receptors may have potential therapeutic usefulness in the treatment of migraine. In addition, a new nonpeptide CGRP antagonist, SB-273779, which blocked the CGRP-induced hypotension in anaesthetised rats (Aiyar *et al.*, 2001), represents an opportunity to analyse its potential antimigraine activity.

Lastly, it has been shown that lanepitant (Goldstein *et al.*, 2001a) and RPR 100893-201 (Diener *et al.*, 1995), very potent substance P (NK$_1$) receptor antagonists, were not clinically effective in aborting migraine (Diener *et al.*, 1995; Goldstein *et al.*, 2001a), although they inhibited the neurogenic dural inflammation. Thus, these results suggest that blockade of NK$_1$ receptors is not a fruitful strategy to relieve migraine.

(V)   AGONISTS AT SPECIFIC α-ADRENORECEPTOR SUBTYPES

It has been shown that specific α_1- and α_2-adrenoceptor subtypes mediate vasoconstriction in the carotid circulation of anaesthetised dogs and pigs, implying that selective agonists at these receptors may be effective in aborting migraine attacks (Willems *et al.*, 2003). Considering the possibility of α_1-adrenoceptor subtypes, it is reported that the α_{1B}-adrenoreceptor subtype does not seem to play an important role in cardiovascular regulation (Willems *et al.*, 2003). Thus, a selective α_{1B}-adrenoceptor agonist may have advantages over the currently available acute antimigraine drugs, which all constrict the human isolated coronary artery (Maassen VanDenBrink *et al.*, 1999); significantly, in this context, α_{1B}-adrenoceptors are not present in the latter vascular bed (Rudner *et al.*, 1999). The possible antimigraine efficacy of selective agonists at specific α_2-adrenoceptor subtypes can be considered on the basis of our findings showing that α_{2C}-adrenoceptors mediate canine external carotid vasoconstriction.   Moreover, α_{2C}-adrenoceptors   exclusively   mediate   the antinociceptive effect of α_2-adrenoceptor agonists (such as clonidine) in rats (Khasar *et al.*, 1995). This property, together with the fact that these receptors mainly mediate vasoconstriction in both anaesthetised pigs (Willems et al., unpublished observations) and dogs (Willems *et al.*, 2001), favours the potential usefulness of selective α_{2C}-adrenoceptor agonists in migraine therapy (Willems *et al.*, 2003). Admittedly, the above findings are mainly based on the effects of antagonists and, therefore, it is crucial to develop potent and selective agonists at α_{1B}- and α_{2C}-adrenoceptor subtypes as potential antimigraine agents.

(VI)   SELECTIVE ADENOSINE A$_1$ RECEPTOR AGONISTS

GR79236, a selective adenosine A$_1$ receptor agonist, inhibits trigeminal nerve firing and calcitonin gene-related peptide release without producing vasoconstriction

(Goadsby *et al.*, 2002b; Giffin *et al.*, 2003). Therefore, GR79236 may have therapeutic potential in migraine and it would be interesting to investigate in migraine patients

(VII)   NO SYNTHESIS INHIBITORS

Several lines of evidence have shown that NO may play a pivotal role in migraine pain (Goadsby *et al.*, 2002b). Moreover, migraineurs are hypersensitive to nitroglycerin, and the NO donor nitroglycerin (glyceryl trinitrate) triggers genuine migraine attacks (Olesen *et al.*, 1994). Apart from potent vasodilating effects, NO: (i) mediates central processing of pain by interacting with central nervous system NMDA (N-methyl-D-aspartate) receptors (Hibbs *et al.*, 1988; Kolesnikov *et al.*, 1992); and (ii) causes CGRP release from perivascular nerve endings (Strecker *et al.*, 2002; Strecker & Messlinger, 2003). Therefore, a substance that inhibits NO production may be a useful in acute migraine treatment (Lassen *et al.*, 1997; Goadsby *et al.*, 2002b). This hypothesis was explored by studying the effect of NOS inhibitor, L-N^a-methylarginine hydrochloride (546C88) in migraine patients; the study reported that NOS inhibitor was effective in headache relief (Lassen *et al.*, 1997). However, the results are from a small group of patients and further clinical investigations are warranted to prove the efficacy of NOS inhibitors in migraine therapy.

(VIII)   OTHER POSSIBLE AVENUES

There are several agents that are explored for antimigraine potential. Among those: (i) civamide (chemically related to capsaicin) (Doggrell, 2001), a vanilloid receptor agonist (ii) neuroactive steroids (e.g., ganaxolone) (Ramadan, 2001); and (iii) octreotide (Kapicioglu *et al.*, 1997), a somatostatin analogue have shown promising results in clinical investigations.

Finally, to attain a maximal therapeutic efficacy, the antimigraine compounds should act via several mechanisms (De Vries *et al.*, 1999a). However, an antimigraine agent, which elicits its effect on the trigeminal system without producing cranial vasoconstriction, may not be effective in acute migraine management. Therefore, the future goal is to focus more on the development of selective vasoconstrictor compounds with minor side effects.

6.9   Conclusion

In conclusion, the results of the present thesis show that: (i) BIBN4096BS is a potent CGRP receptor antagonist devoid of important effects *per se* in the cardiovascular system; (ii) endogenous CGRP does not play an important role in regulating the basal vascular tone in anaesthetised rats and pigs; (iii) CGRP receptors are widely distributed in the cardiovascular system of anaesthetised rats; (iv) the antimigraine potential of BIBN4096BS involves CGRP receptor antagonism and (v) the therapeutic action of sumatriptan is mainly due to cranial vasoconstriction rather than trigeminal (CGRP release) inhibition.

CHAPTER 7

Summary

7   SUMMARY

7.1   Summary in English

Chapter 1 is divided into three sections, namely: (i) migraine (Part I); (ii) the putative role of CGRP in migraine pathophysiology (Part II); and (iii) aims of this thesis (part III).

Part I discusses important aspects involved in migraine pathophysiology. Firstly, the historical perspective, epidemiology, clinical features and diagnostic criteria (ICHD'2004) of migraine have been described shortly. Thereafter, based on the current developments in migraine research, the pathophysiology of migraine is explained. Subsequently, the treatment methods (acute and preventive treatments) currently used to relieve migraine are discussed. Lastly, the pharmacology and relevance of the currently used experimental models, which are believed to be predictive of therapeutic potential, have been described in detail.

Part II gives an overview about CGRP, namely: (i) discovery; (ii) structure and distribution; (iii) biological functions; and (iv) receptors. Details regarding CGRP receptor classification, structure, distribution and signal transduction mechanisms were given. This was followed by the therapeutic potentials of CGRP receptor agonists and antagonists. Chapter 2 rounds off with: (i) the potential role of CGRP in the migraine pathophysiology; and (ii) newly developed CGRP receptor antagonists and their potential role as antimigraine drugs.

Part III of Chapter 1 sets out the main aims of this thesis.

In **Chapter 2**, we investigated the effects of BIBN4096BS on capsaicin-induced porcine carotid haemodynamics and on plasma CGRP release. BIBN4096BS has recently been introduced as an experimental tool (antagonist) to investigate the CGRP receptor-mediated functional responses. Our results in anaesthetised pigs show that: (i) i.c. administration of capsaicin dilated carotid arteriovenous anastomoses and arterioles, together with an increase in carotid pulsations and a narrowing of the A-V SO_2 difference as well as an elevation of jugular venous plasma CGRP concentrations; and (ii) BIBN4096BS dose-dependently antagonised the changes in carotid haemodynamics and A-V SO_2 difference caused by capsaicin, but it enhanced the capsaicin-induced increase in jugular venous plasma CGRP concentration. Taken together, these results suggest that: (i) CGRP receptors are present (and operative) in

the carotid circulation; and (ii) BIBN4096BS behaves as an antagonist of capsaicin-induced carotid haemodynamic changes that are mediated via the release of CGRP.

In **Chapter 3**, we have investigated the effects of BIBN4096BS on the cardiac output distribution and on α-CGRP induced porcine carotid haemodynamics in a model predictive of antimigraine activity. In cardiac output experiments, BIBN4096BS (100, 300 and 1000 μg kg^{-1}; i.v) treatment did not alter the systemic and regional haemodynamics. In carotid haemodynamic experiments, BIBN4096BS dose-dependently antagonised α-CGRP-induced: (i) decrease in mean blood pressure; (ii) increase in carotid and arteriovenous anastomotic conductance; and (iii) A-V SO$_2$ difference. The above findings indicate that: (i) BIBN4096BS behaves as a CGRP receptor antagonist on the porcine systemic and carotid circulation; and (ii) endogenous CGRP does not play a significant role in the regulation of basal vascular tone on the basis of the failure of BIBN4096BS to modify systemic and regional haemodynamics per se.

Chapter 4 investigates in anaesthetised pigs the effects of sumatriptan on capsaicin-induced carotid haemodynamic changes and on plasma CGRP release. It was demonstrated that infusions of capsaicin: (i) increased total carotid, arteriovenous anastomotic and capillary blood flows and conductances; (ii) narrowed the A-V SO$_2$ difference; and (iii) increased plasma CGRP concentrations. These capsaicin-induced responses (except those in arteriovenous anastomotic blood flow and conductance where sumatriptan produces vasoconstriction) were not modified by sumatriptan. Our results support the contention that the therapeutic action of sumatriptan is mainly due to cranial vasoconstriction rather than trigeminal (CGRP release) inhibition.

In **Chapter 5**, we investigated in anaesthetised rats the effects of BIBN4096BS on α-CGRP-induced systemic and regional haemodynamic changes. Our findings show that α-CGRP infusions: (i) decreased mean arterial blood pressure and increased heart rate as well as systemic vascular conductance; (ii) increased the vascular conductances to the heart, brain, gastrointestinal tract, adrenals, skin and skeletal muscles. The above α-CGRP-induced responses were attenuated by BIBN4096BS. Moreover, BIBN4096BS pre-treatment did not alter the baseline systemic and regional

haemodynamics. These lines of evidence indicate that: (i) exogenously administered α-CGRP dilates the regional vascular beds via CGRP receptors on the basis of the antagonism produced by BIBN4096BS; (ii) endogenously produced CGRP does not play an important role in regulating the systemic and regional haemodynamics under resting conditions; and (iii) BIBN4096BS has less affinity for rat CGRP receptors.

7.2   Samenvatting in het Nederlands (Summary in Dutch)

Hoofdstuk Één is onderverdeeld in drie delen, namelijk: (i) migraine (Deel I); (ii) mogelijke rol van calcitonin gene-related peptide (CGRP) in migraine pathofysiologie (Deel II), and (iii) doelen van huidig proefschrift (Deel III).

Deel I beschrijft belangrijke aspecten over het the ontstaan (pathofysiologie) van migraine. Historische perspectieven, epidemiologie, klinische aspecten en diagnostische criteria (IHS 2004) van migraine worden in het kort beschreven. Gebaseerd op recente bevindingen in migraine onderzoek, wordt de pathofysiologie en mogelijke behandelingen (acuut en preventief) van migraine beschreven. Ten slotte wordt de farmacologie en de mogelijk voorspelbare waarde van experimentele modellen in detail beschreven.

Deel II geeft een beknopte uiteenzetting over CGRP, namelijk: (i) ontdekking; (ii) structuur en distributie; (iii) biologische functies; en (iv) receptoren. Details omtrent CGRP receptoren (classificatie, structuur, distributie en signaal-transductie mechanismen) worden beschreven, gevolgd door mogelijk therapeutische mogelijkheden van CGRP receptor agonisten (receptor stimulatie) en antagonisten (receptor blokkade). Het hoofdstuk wordt afgesloten door een beschrijving van CGRP in migraine pathogenesis (het ontstaan) en nieuwe CGRP receptor antagonisten als mogelijke antimigraine drugs.

Deel III beschrijft de doelen van het proefschrift.

In **Hoofdstuk Twee** worden de hematologische (bloeddoorstroming) effecten van BIBN4096BS, alsmede de effecten op CGRP afgifte, na toediening van capsaicin (belangrijk ingrediënt van rode pepers) in genarcotiseerde varkens onderzocht. Het is recentelijk aangetoond dat BIBN4096BS kan worden gebruikt om de betrokkenheid van CGRP receptoren te onderzoeken. Onze resultaten laten zien dat: (i) lokale toediening van capsaicin in het halsslagader (carotid) vaatbed veroorzaakt relaxatie

(dilatatie) van halsslagader arterioveneuze anastomoses en arteriolen, alsmede een toename in halsslagader pulsaties, een verlaging van het verschil in arterieele en jugulair-veneuze zuurstofverzadiging (A-V SO_2) en een verhoging van de veneuze plasma CGRP concentraties; en (ii) BIBN4096BS verminderde de hematologische capsaicin-geïnduceerde veranderingen in het halsslagader vaatbed en A-V SO_2 verschil. Echter, BIBN4096BS veroorzaakte een verhoging van CGRP plasma concentratie. De effecten van BIBN4096BS waren dosisafhankelijk. Deze resultaten suggereren dat BIBN4096BS een antagonist is van capsaicin-geïnduceerde carotid hematologische veranderingen, die veroorzaakt worden via de release van CGRP.

In **Hoofdstuk Drie** hebben we de effecten bestudeerd van BIBN4096BS op de distributie van de cardiac output, alsmede op humaan α-CGRP geïnduceerd hematologische effecten in een varkensmodel met voorspellende waarde voor migraine activiteit. BIBN4096BS toediening (100, 300 and 1000 μg kg^{-1}; i.v) veroorzaakte geen systemisch of regionaal hematologische veranderingen in de cardiac output experimenten. Echter, in de carotid experimenten veroorzaakte BIBN4096BS een dosisafhankelijke verlaging van de α-CGRP geïnduceerde: (i) verlaging in bloeddruk (hypotensie); (ii) verhoging in de conductance van het halsslagader vaatbed en arterioveneuze anastomoses; en van het (iii) A-V SO_2 verschil. Bovengenoemde bevindingen suggereren dat: BIBN4096BS een CGRP receptor antagonist is in zowel het systemische als halsslagader circulatie in varkens; en dat (ii) endogeen (lichaamseigen) CGRP geen belangrijke rol speelt in de regulatie van de basaal vasculaire tonus, omdat BIBN4096BS per se geen systemische of regionale veranderingen teweegbracht.

Hoofdstuk Vier onderzocht de mogelijke effecten van het veel gebruikte antimigraine middel sumatriptan op de capsaicin-geïnduceerde veranderingen in bloeddoorstroming en systemische CGRP afgifte in het halsslagader vaatbed van genarcotiseerde varkens. Toediening van capsaicin veroorzaakte: (i) een toename in halsslagader vaatbed bloeddoorstroming (en conductance) en in corresponderende distributie (arterioveneuze anastomoses en capillaire bloedvaten); (ii) een verlaging van het A-V SO_2 verschil; en (iii) een verhoging van plasma CGRP concentraties. Deze capsaicin-geïnduceerde effecten [behalve die in arterioveneuse anastomotische bloeddoorstroming en conductance, waar sumatriptan een constrictie (=samentrekken

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van bloedvaten) veroorzaakt] bleven onveranderd na toediening van sumatriptan. Onze resultaten bevestigen de hypothese dat de therapeutische effectiviteit van sumatriptan in migraine wordt voornamelijk veroorzaakt door een selectieve craniële (in halsslagader circulatie) vasoconstrictie en niet (of in minder mate) door de remming van CGRP afgifte uit (trigeminal) zenuwuiteinden.

In **Hoofdstuk Vijf** hebben we de effecten van BIBN4096BS bestudeerd op de systemisch en regionaal hematologische veranderingen na toediening van humaan α-CGRP in genarcotiseerde ratten. Onze bevindingen laten zien dat α-CGRP toediening produceert: (i) hypotensie en tachycardie, alsmede een verhoging van de systemisch vasculaire conductance (bloeddoorstroming gecorrigeerd voor bloeddrukveranderingen); (ii) een toename in regionaal vasculaire conductance naar verschillende goed doorbloede weefsels/organen, inclusief het hart, hersenen, darmen, huid en skeletspieren. Bovengenoemd α-CGRP-geïnduceerde effecten waren verminderd door BIBN4096BS. Bovendien, behandeling van de ratten met BIBN4096BS veroorzaakte geen verandering in systemische and regionale hematologische parameters (bijvoorbeeld bloeddoorstroming en hartritme) per se. Deze resultaten leveren bewijs dat: (i) exogeen (niet-lichaamseigen) toegediende α-CGRP produceert dilatatie (relaxatie) in specifieke vaatbedden via CGRP receptoren; (ii) endogeen (lichaamseigen) geproduceerd CGRP speelt geen belangrijke rol in basaal systemisch of basaal regionaal hematologische parameters in genarcotiseerde ratten; en (iii) BIBN4096BS heeft een lagere affiniteit voor rat CGRP receptoren in vergelijking humane CGRP receptoren.

CHAPTER 8

Appendix

8   APPENDIX

8.1   Acknowledgement

I consider my work is incomplete without this section. To begin with, I would like to acknowledge NIHES and NUFFIC for providing me the gateway to The Netherlands.

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8.2   About the author

Udayasankar Arulmani was born in Madras, India on 30th July 1973. He did his secondary and higher secondary school education in Madras Christian College Higher secondary school, Madras, India.   After passing out in 1991, he joined Madras Medical College and Research Institute for his bachelor degree in medicine (MBBS). He obtained his bachelor degree in medicine in 1997.   He then worked as senior resident in the Department of Rheumatology and Immunology, Government General Hospital, Madras for a year. In august 2000, he was awarded a fellowship from The Netherlands Organisation for International Cooperation in Higher Education (NUFFIC) for pursuing his Masters degree in Clinical Epidemiology, in The Netherlands Institute for health sciences (NIHES), Erasmus Medical Centre, Rotterdam.   During his master's programme, he developed a decision-cost effectiveness model for the treatment of renal artery stenosis under the guidance of Prof. Myrium Hunink. He obtained his Maters degree in Clinical Epidemiology in August 2001. Thereafter, he initiated his research career with a project entitled "The role of CGRP in the pathophysiology of migraine" under the supervision of Prof. Dr. Pramod R. Saxena. During this period he also had an opportunity to do collaborative projects with Prof. Dr. Carlos .M. Villalón (Departamento de Farmacología y Toxicología, Mexico D.F).

8.3   Publications

Full papers

1. Arulmani U, Heiligers JP, Garrelds IM, Sanchez-Lopez A, Willems EW, Villalón CM and Saxena PR. Effects of sumatriptan on capsaicin-induced carotid haemodynamic changes and CGRP release in anaesthetised pigs. Cephalalgia (in press) 2003.

2. Arulmani U, Schuijt MP, Heiligers JP, Garrelds IM, Willems EW, Villalón CM, and Saxena PR. Effects of the CGRP receptor antagonist BIBN4096BS on CGRP-induced regional haemodynamic changes in anaesthetised rats. Pharmacol&Toxicol (in press) 2003.

3. Kapoor K, Arulmani U, Heiligers JP, Garrelds IM, Willems EW, Doods H, Villalón CM and Saxena PR. Effects of the CGRP receptor antagonist BIBN4096BS on capsaicin-induced carotid haemodynamic changes in anaesthetised pigs. Br J Pharmacol 2003; 140:329-38.

4. Kapoor K, Arulmani U, Heiligers JP, Willems EW, Doods H, Villalón CM and Saxena PR. Effects of BIBN4096BS on cardiac output distribution and on CGRP-induced carotid haemodynamic responses in the pig. Eur J Pharmacol 2003; 475:69-77.

5. Sanchez-Lopez A, Centurión D, Vazquez E, Arulmani U, Saxena PR and Villalón CM. Pharmacological profile of the 5-HT-induced inhibition of cardioaccelerator sympathetic outflow in pithed rats: correlation with 5-HT1 and putative 5-ht5A/5B receptors. Br J Pharmacol 2003; 140:725-35.

6. Sanchez-Lopez A, Centurión D, Vazquez E, Arulmani U, Saxena PR and Villalón CM. Further characterization of the 5-HT_1 receptors mediating cardiac sympatho-inhibition in pithed rats: pharmacological correlation with the 5-HT_{1B} and 5-HT_{1D} subtypes. Naunyn Schmiedebergs Arch Pharmacol 2004; 369:220-7.

7. Valdivia LF, Centurión D, Perusquia M, Arulmani U, Saxena PR and Villalón CM. Pharmacological analysis of the mechanisms involved in the tachycardic and vasopressor responses to the antimigraine agent, isometheptene, in pithed rats. Life Sci 2004; 74:3223-34.

8.   Villalon CM, Centurion D, Willems EW, Arulmani U, Saxena PR and Valdivia LF. 5-HT$_{1B}$ receptors and alpha (2A/2C)-adrenoceptors mediate external carotid vasoconstriction to dihydroergotamine. Eur J Pharmacol 2004; 484:287-90.

9.   Chellapandian D, Vijay Kumar TA, Arulmani U, Panchapakesa Rajendran C, Porkodi R, Madhavan. R and Chandra Sekaran AN. Male Lupus -A Retrospective Analysis of Clinical and Laboratory Features in 32 Patients. J Ind Rheum Ass 1998; 6:25-8.

10. Vijay Kumar TA, Arulmani U, Chellapandian D, Panchapakesa Rajendran C, Kanagarani P, Parthiban M and Chandra Sekaran AN. Late Onset Systemic lupus Erythematosus. J Ind Rheum Ass 1998; 6:35-7.

11. Arulmani U, Heiligers JP, Valdivia LP, Garrelds IM, Villalón CM and Saxena PR. Lack of effect of Adenosine (A$_1$) receptor agonist, GR79236 on capsaicin-induced CGRP release in anaesthetised pigs (submitted to Cephalalgia) 2004.

12. Arulmani U, MaassenVanDenBrink A, Villalón CM and Saxena PR. Putative role of calcitonin gene-related peptide in migraine pathophysiology-a comprehensive review (submitted to Eur J Pharmacol) 2004.

13. Krijnen P, van Helvoort-Postulart D, Arulmani U, van Dijk L, van Jaarsveld B, Hunink MG. Cost-effectiveness of treatment strategies for hypertensive patients with renal artery stenosis on MRA or CTA (submitted to JAMA) 2004.

14. Valdivia LP, Centurión D, Arulmani U, Saxena PR and Villalón CM. 5-HT$_{1B}$ receptors, a$_{A/2C}$- and, to a lesser extent, a$_1$-arenoceptors mediate the external carotid vasoconstriction to ergotamine in vagosympathectomized dogs (submitted to Naunyn Schmiedebergs Arch Pharmacol) 2004

15. Arulmani U, Willems EW, Heiligers JP, Valdivia LP, Villalón CM and Saxena PR. The effects of alpha (2)-adrenoceptor agonist (BHT933) on capsaicin-induced CGRP release and the role of alpha (2)-adrenoceptor subtypes on carotid vasoconstriction in anaesthetised pigs (in preparation) 2004.

16. Arulmani U, MaassenVanDenBrink A, Villalón CM and Saxena PR. Experimental migraine models and their relevancy in migraine therapy- review (in preparation) 2004

Book chapters

1. Arulmani U, Villalon CM, Saxena PR. Effects of BIBN4096BS on capsaicin-induced haemodynamic changes in a porcine experimental model for migraine. In: Couturier E, ed. Anglo-Dutch Migraine Association Proceeding. Rotterdam: The Netherlands, 2004 (in press)

2. MaassenVanDenBrink A, Arulmani U, Saxena PR. Role of CGRP and CGRP receptor antagonists in migraine. In: Couturier E, ed. Anglo-Dutch Migraine Association Proceeding, Chester, 2005 (in press)

Abstracts

1. Arulmani U., Heiligers JP, Garrelds IM, Sanchez-Lopez A, Willems EW, Villalón CM and Saxena PR, 2004. Effects of sumatriptan on capsaicin-induced carotid haemodynamic changes and CGRP release in anaesthetised pigs, European Headache Federation congress, Rotterdam, The Netherlands.

2. Arulmani U, Kapoor K, Heiligers JP, Garrelds IM, Willems EW, Doods H, Villalón CM and Saxena PR, 2003a. Effects of BIBN4096BS on capsaicin-induced haemodynamic changes in a porcine experimental model for migraine, Pfizer Drug Discovery. Vol. 140 (Pfizer, Sandwich, United Kingdom) p. 329-338.

3. Arulmani U, Kapoor K, Heiligers JP, Garrelds IM, Willems EW, Doods H, Villalón CM and Saxena PR, 2003b. Effects of BIBN4096BS on capsaicin-induced haemodynamic changes in anaesthetised pigs, XI Congress of the International headache society/ *IHC 2003*. Vol. 23 (Cephalalgia, Rome, Italy) p. 729-730.

4. Arulmani U, Kapoor K, Heiligers JP, Garrelds IM, Willems EW, Doods H, Villalón CM and Saxena PR, 2003c. Effects of BIBN4096BS on regional cardiac output and on a-CGRP-induced carotid haemodynamic changes in a anaesthetised pigs, XI Congress of the International headache society/ *IHC 2003*. Vol. 23 (Cephalalgia, Rome, Italy) p. 727.

5. Arulmani U, Kapoor K, Heiligers JP, Willems EW, Doods H, Villalón CM and Saxena PR, 2003d. Effects of BIBN4096BS on capsaicin-induced

haemodynamic changes in a porcine experimental model for migraine, in: Farmacologiedagen 2003Lunteren, The Netherlands).

6.   Sanchez-Lopez, A., Centurión D, Vazquez E, Arulmani U, Saxena PR and Villalón CM, 2004. Characterization of the 5-HT1 receptors mediating cardiac sympatho-inhibition in pithed rats: pharmacological correlation with the 5-HT$_{1B}$ and 5-HT$_{1D}$ subtypes, Serotonin EPHAR Satellite Meeting, Porto.

7.   Valdivia, L.P, Arulmani U, Saxena PR and Villalón CM, 2004. Evidence that 5-HT$_{1B}$ receptors and alpha 2A/2C-adrenoreceptors mediate the external carotid vasoconstriction to ergotamine in vagosympathectomised dogs, Serotonin EPHAR Satellite Meeting, Porto.

8.   Villalón CM, Centurión D, Willems EW, Arulmani U, Saxena PR and Valdivia LF, 2003. The role of 5-HT$_{1B}$ receptors as well as alpha 2A and alpha 2C adrenoceptor subtypes mediating the canine external carotid vasoconstriction to dihydroergotamine, American Headache Society, Florida, USA.

8.4   List of abbreviations

°C:	Degrees Celsius
4991w93:	4-[3-(trans-3-dimethylaminocyclobutyl)-1H-indol-5-ylmethyl]-(4S)-oxazolidi n-2-one
546C88:	L-N^a-methylarginine hydrochloride
5-HT:	5-hydroxytryptamine
a.u.:	Arbitary units
AD:	Anno Domini
ATP:	Adenylate tri phosphate
A-V SO$_2$ difference:	Difference between arterial and jugular venous oxygen saturations
BC:	Before Christ
BIBN4096BS:	1-Piperidinecarboxamide, N-[2-[[5-amino-1-[[4-(4-pyridinyl)-1-piperazinyl]carbonyl]pentyl]amino]-1-[(3,5-dibromo-4-hydroxyphenyl)methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2H)-quinazolinyl)
cAMP:	Cyclic adenosine monophosphate
cDNA:	Complimentry DNA
Ce:	Cerium
c-Fos:	FBJ osteosarcoma oncogene
cGMP:	Cyclic guanosine 5'- monophosphate
CGRP:	Calcitonin gene related peptide
CNS:	Central nervous system
COL-29:	Human colonic epithelium-derived cell line
Compound 1:	(4-(2-Oxo-2,3-dihydro-benzoimidazol-1-yl)-piperidine-1-carboxylic acid [1-(3,5-dibromo-4-hydroxy-benzyl)-2-oxo-2-(4-phenyl-piperazin-1-yl)-ethyl] -amide)
CP122288:	5-methylaminosulphonylmethyl-3-(N-methoxy-pyrrolidin-2R-yl-methyl)-1H-indole
CRLR:	Calcitonin receptor-like receptor
Cys(ACM)^{2,7}]hα-CGRP:	[acetamidomethyl-cysteine^{2,7}]CGRPα;
Cys(Acm)^{2,7}hα-CGRP:	[acetamidomethyl-cysteine^{2,7}]CGRPα

DNA:	deoxy ribonucleic acid
e.g.:	For example
et al.:	and colleagues
ET:	Endothelin
FBJ:	Finkel-Biskis-Jinkins
FHM:	Familial hemiplegic migraine
GPCR:	G-protein coupled receptor
GR127935:	N-[methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl) [1,1-biphenyl]-4-carboxamide hydrochloride
GR79236:	N-[(2-methylphenyl)methyl]adenosine (metrifudil), 2-(phenylamino)adenosine (CV1808),
h:	Hour(s)
HCA-7:	Human colonic epithelium-derived cell line
HEK293:	Human embryonic kidney cells
i.c.:	Intracarotid route of administration
i.m.:	Intramuscular
i.p.:	Intraperitoneal route of administration
i.v.:	Intravenous route of administration
i-CGRP:	immunoreactive CGRP
ICHD:	International classification of headache disorders
IHS:	International Headache Society
IS159:	3-(2-aminoethyl)-5-[acetamidyl-3-(4-hydroxyphenyl)-propionamidyl-acetamidyl-oxy]-indole
IUPHAR:	International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification
K^+:	Potassium ions
KeV:	Kilo electro-volt (radioactive γ-radiation)
kg:	Kilogram(s) (10^3 gram)
KIU:	Kininogenase inhibitor units
L6:	A cell line representative of skeletal muscle
LY334370:	4-fluoro-N-[3-(1-methyl-4-piperidinyl)-1H-indol-5-yl]-benzamide

LY344864:	N-[3-(dimethylamino)-2,3,4,9-tetrahydro-1H-carbazol-6-yl]-4-fluorobenzamide
min:	Minute(s)
ml:	Milliliter(s)
mmHg:	Millimeter mercury
MRI:	Magnetic resonance imaging
mRNA:	messenger ribonucleic acid
n:	Number of animals used
NaoH:	Sodium hydroxide
Nb:	Niobium
NIHES:	The Netherlands Institute for Health Sciences
NK:	Neurokinin
nM:	Nanomolar
NMDA:	N-Methyl-D-aspartate
NO:	Nitric oxide
NOS:	Nitric oxide synthase
NSAID:	Non-steroidal antiinflammatory drugs
NUFFIC:	The Netherlands Organisation for International Cooperation in Higher Education
pA_2:	Negative logarithm to base 10 of the molar concentration of the antagonist that makes it necessary to double the concentration of the agonist needed to elicit the original submaximal response.
pg:	Picogram
pH:	Negative logarithm to base 10 of the hydrogen (H) concentration
pK_i:	Negative logarithm of a concentration of a competing ligand in a competition assay that would occupy 50% of the receptors in no radioligand would be present
pM:	Picomolar
PNU109291:	(s)-3,4-dihydro-1-ethyl]-N-methyl-1H-2-benzopyran-6-carboximide

PNU-142633:	(s)-3,4-dihydro-1-[2-[4-[4-aminocarbonyl) phenyl]-1-piperazinyl]ethyl]-N-methyl-1H-2-benzopyran-6-carboximide
PNU-142633F:	(s)-3,4-dihydro-1-[2-[4-[4-aminocarbonyl) phenyl]-1-piperazinyl] ethyl]-N-methyl-1H-2-benzopyran-6-carboximide
poly A:	Polyadenylation
RAMP:	Receptor activity modifying protein
RBI:	Research Biochemicals International (SIGMA-Aldrich)
RCP:	Receptor component protein;
RIA:	Radioimmunoassay
RP 67580:	2-[1-amino-2-(2-methoxy phenyl) ethyl]-7,7 diphenyl-4 perhydro-isoindolone-(3aR,7aR)
RRA:	Radioreceptor assay
Ru:	Ruthenium
S.D.:	Standard deviation
s.e.m.:	Standard error of the mean
SB-(+)-273779:	[N-methyl-N-(2-methylphenyl)-3-nitro-4-(2-thiazolylsulfinyl)nitrobenzanilide]
SB224289:	2,3,6,7-tetrahydro-1'-methyl-5-[2'-methyl-4'(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-carbonyl] furo [2,3-f] indole-3-spiro-4`-piperidine hydrochloride
Sc:	Scandium
SK-N-MC cells:	Human neuroblastoma cell line;
Sn:	Tin
SPSS:	Statistical Package for Social Sciences
SVC:	Systemic vascular conductance
U.S.A.:	United States of America
VIP:	Vasoactive intestinal peptide
vs.:	Verses
μg:	Microgram (10^{-6} g)

8.5   References

Ackermann, P.W., Ahmed, M. & Kreicbergs, A. (2002). Early nerve regeneration after achilles tendon rupture--a prerequisite for healing? A study in the rat. *J Orthop Res*, **20,** 849-856.

Aiyar, N., Daines, R.A., Disa, J., Chambers, P.A., Sauermelch, C.F., Quiniou, M., Khandoudi, N., Gout, B., Douglas, S.A. & Willette, R.N. (2001). Pharmacology of SB-273779, a nonpeptide calcitonin gene-related peptide 1 receptor antagonist. *J Pharmacol Exp Ther*, **296,** 768-775.

Aiyar, N., Rand, K., Elshourbagy, N.A., Zeng, Z., Adamou, J.E., Bergsma, D.J. & Li, Y. (1996). A cDNA encoding the calcitonin gene-related peptide type 1 receptor. *J Biol Chem*, **271,** 11325-11329.

Akerman, S., Kaube, H. & Goadsby, P.J. (2003). Vanilloid type 1 receptors (VR1) on trigeminal sensory nerve fibres play a minor role in neurogenic dural vasodilatation, and are involved in capsaicin-induced dural dilation. *Br J Pharmacol*, **140,** 718-724.

Akerman, S., Williamson, D.J., Kaube, H. & Goadsby, P.J. (2002). Nitric oxide synthase inhibitors can antagonize neurogenic and calcitonin gene-related peptide induced dilation of dural meningeal vessels. *Br. J. Pharmacol.*, **137,** 62-68.

Alevizaki, M., Shiraishi, A., Rassool, F.V., Ferrier, G.J., MacIntyre, I. & Legon, S. (1986). The calcitonin-like sequence of the beta CGRP gene. *FEBS Lett*, **206,** 47-52.

Alvarez-Cermeno, J., Gobernado, J.M. & Aimeno, A. (1986). Transient blood-brain barrier (BBB) damage in migraine. *Headache*, **26,** 437.

Alving, K. & Franco-Cereceda, A. (1993). The ability of ruthenium red to reduce the autonomic reflexes and peptide release evoked by capsaicin administration in the pig *in vivo*. *Acta. Physiol. Scand*, **147,** 315-321.

Alving, K., Matran, R. & Lundberg, J.M. (1991). Capsaicin-induced local effector responses, autonomic reflexes and sensory neuropeptide depletion in the pig. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **343,** 37-45.

Amara, S.G., Arriza, J.L., Leff, S.E., Swanson, L.W., Evans, R.M. & Rosenfeld, M.G. (1985). Expression in brain of a messenger RNA encoding a novel neuropeptide homologous to calcitonin gene-related peptide. *Science*, **229,** 1094-1097.

Amara, S.G., Jonas, V., Rosenfeld, M.G., Ong, E.S. & Evans, R.M. (1982). Alternative RNA processing in calcitonin gene expression generates *mRNA*s encoding different polypeptide products. *Nature*, **298,** 240-244.

Andermann, F. (1987). Migraine-epilepsy relationships. *Epilepsy Res*, **1,** 213-226.

Arden, W.A., Fiscus, R.R., Wang, X., Yang, L., Maley, R., Nielsen, M., Lanzo, S. & Gross, D.R. (1994). Elevations in circulating calcitonin gene-related peptide correlate with haemodynamic deterioration during endotoxic shock in pigs. *Circ. Shock*, **42,** 147-153.

Asari, J., Suzuki, K., Matsumoto, M., Sasaki, T. & Kodama, N. (2001). Antidromic effect of calcitonin gene-related peptide containing nerves on cerebral arteries in rats - a possible role of sensory nerves on cerebral circulation. *Fukushima. J. Med. Sci.*, **47,** 75-84.

Ashina, M., Bendtsen, L., Jensen, R., Schifter, S. & Olesen, J. (2000). Evidence for increased plasma levels of calcitonin gene-related peptide in migraine outside of attacks. *Pain*, **86,** 133-138.

Ashkenazi, A. & Silberstein, S.D. (2003). The evolving management of migraine. *Curr Opin Neurol*, **16,** 341-345.

Bahra, A., Matharu, M.S., Buchel, C., Frackowiak, R.S. & Goadsby, P.J. (2001). Brainstem activation specific to migraine headache. *Lancet*, **357,** 1016-1017.

Baile, E.M., Nelems, J.M., Schulzer, M. & Pare, P.D. (1982). Measurement of regional bronchial arterial blood flow and bronchovascular resistance in dogs. *J. Appl. Physiol.*, **53,** 1044-1049.

Bard, J.A., Zgombick, J., Adham, N., Vaysse, P., Branchek, T.A. & Weinshank, R.L. (1993). Cloning of a novel human serotonin receptor (5-HT7) positively linked to adenylate cyclase. *J Biol Chem*, **268,** 23422-23426.

Beattie, D.T. & Connor, H.E. (1994). The influence of the trigeminal ganglion on carotid blood flow in anaesthetized guinea-pigs. *Br J Pharmacol*, **112,** 262-266.

Bell, D. & McDermott, B.J. (1996). Calcitonin gene-related peptide in the cardiovascular system: characterization of receptor populations and their (patho)physiological significance. *Pharmacol. Rev.*, **48,** 253-288.

Bhalla, P., Sharma, H.S., Ma, X., Wurch, T., Pauwels, P.J. & Saxena, P.R. (2001). Molecular cloning, pharmacological properties and tissue distribution of the porcine 5-HT$_{1B}$ receptor. *Br. J. Pharmacol.*, **133,** 891-901.

Bhalla, P., Sharma, H.S., Wurch, T., Pauwels, P.J. & Saxena, P.R. (2002). Molecular cloning and expression of the porcine trigeminal ganglion cDNA encoding a 5-ht$_{1F}$ receptor. *Eur. J. Pharmacol.*, **436,** 23-33.

Bigal, M.E., Rapoport, A.M., Sheftell, F.D. & Tepper, S.J. (2002). New migraine preventive options: an update with pathophysiological considerations. *Rev Hosp Clin Fac Med Sao Paulo*, **57,** 293-298.

Bivalacqua, T.J., Champion, H.C., Abdel-Mageed, A.B., Kadowitz, P.J. & Hellstrom, W.J. (2001). Gene transfer of prepro-calcitonin gene-related peptide restores erectile function in the aged rat. *Biol Reprod*, **65,** 1371-1377.

Bouchelet, I., Case, B., Olivier, A. & Hamel, E. (2000). No contractile effect for 5-HT$_{1D}$ and 5-HT$_{1F}$ receptor agonists in human and bovine cerebral arteries: similarity with human coronary artery. *Br J Pharmacol*, **129,** 501-508.

Brain, S.D., Tippins, J.R., Morris, H.R., MacIntyre, I. & Williams, T.J. (1986). Potent vasodilator activity of calcitonin gene-related peptide in human skin. *J Invest Dermatol*, **87,** 533-536.

Brain, S.D. & Williams, T.J. (1985). Inflammatory oedema induced by synergism between calcitonin gene-related peptide (CGRP) and mediators of increased vascular permeability. *Br J Pharmacol*, **86,** 855-860.

Brain, S.D., Williams, T.J., Tippins, J.R., Morris, H.R. & MacIntyre, I. (1985). Calcitonin gene-related peptide is a potent vasodilator. *Nature*, **313,** 54-56.

Brandes, J.L. (2002). Global trends in migraine care: results from the MAZE survey. *CNS Drugs*, **16 Suppl 1,** 13-18.

Brandli, P., Loffler, B.M., Breu, V., Osterwalder, R., Maire, J.P. & Clozel, M. (1996). Role of endothelin in mediating neurogenic plasma extravasation in rat dura mater. *Pain*, **64,** 315-322.

Bratveit, M., Haugan, A. & Helle, K.B. (1991). Effects of calcitonin gene-related peptide (CGRP) on regional haemodynamics and on selected hepato-splanchnic arteries from the rat: a comparison with VIP and atriopeptin II. *Scand J Clin Lab Invest*, **51,** 167-174.

Breslau, N., Davis, G.C. & Andreski, P. (1991). Migraine, psychiatric disorders, and suicide attempts: an epidemiologic study of young adults. *Psychiatry Res*, **37,** 11-23.

Buzzi, M.G., Bonamini, M. & Moskowitz, M.A. (1995). Neurogenic model of migraine. *Cephalalgia*, **15,** 277-280.

Buzzi, M.G., Carter, W.B., Shimizu, T., Heath, H.d. & Moskowitz, M.A. (1991). Dihydroergotamine and sumatriptan attenuate levels of CGRP in plasma in rat superior sagittal sinus during electrical stimulation of the trigeminal ganglion. *Neuropharmacology*, **30,** 1193-1200.

Buzzi, M.G., Dimitriadou, V., Theoharides, T.C. & Moskowitz, M.A. (1992). 5-Hydroxytryptamine receptor agonists for the abortive treatment of vascular headaches block mast cell, endothelial and platelet activation within the rat dura mater after trigeminal stimulation. *Brain Res*, **583,** 137-149.

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D. & Julius, D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature*, **389,** 816-824.

Centurión, D., Ortiz, M.I., Sánchez-López, A., De Vries, P., Saxena, P.R. & Villalón, C.M. (2001). Evidence for 5-HT$_{(1B/1D)}$ and 5-HT$_{(2A)}$ receptors mediating constriction of the canine internal carotid circulation. *Br. J. Pharmacol.*, **132,** 983-990.

Chang, Y., Stover, S.R. & Hoover, D.B. (2001). Regional localization and abundance of calcitonin gene-related peptide receptors in guinea pig heart. *J Mol Cell Cardiol*, **33,** 745-754.

Chantry, A., Leighton, B. & Day, A.J. (1991). Cross-reactivity of amylin with calcitonin-gene-related peptide binding sites in rat liver and skeletal muscle membranes. *Biochem J*, **277 (Pt 1),** 139-143.

Chaveau, J. & Delaage, M.A. (1997). IS-159: a high-affinity specific 5-HT$_{1D}$ full receptor agonist, effective in the acute treatment of migraine. In *Headache treatment: trial methodology and new drugs*. eds OLESEN, J. & TFELT-HANSEN, P. pp. 287-292. New York: Lippincott-Raven publishers.

Chiba, T., Yamaguchi, A., Yamatani, T., Nakamura, A., Morishita, T., Inui, T., Fukase, M., Noda, T. & Fujita, T. (1989). Calcitonin gene-related peptide receptor antagonist human CGRP-(8-37). *Am J Physiol*, **256,** E331-335.

Cohen, Z., Bouchelet, I., Olivier, A., Villemure, J.G., Ball, R., Stanimirovic, D.B. & Hamel, E. (1999). Multiple microvascular and astroglial 5-hydroxytryptamine receptor subtypes in human brain: molecular and pharmacologic characterization. *J Cereb Blood Flow Metab*, **19,** 908-917.

Conner, A.C., Hay, D.L., Howitt, S.G., Kilk, K., Langel, U., Wheatley, M., Smith, D.M. & Poyner, D.R. (2002). Interaction of calcitonin-gene-related peptide with its receptors. *Biochem Soc Trans*, **30,** 451-455.

Coupe, M.O., Mak, J.C., Yacoub, M., Oldershaw, P.J. & Barnes, P.J. (1990). Autoradiographic mapping of calcitonin gene-related peptide receptors in human and guinea pig hearts. *Circulation*, **81,** 741-747.

Critchley, M. (1967). Migraine: from Cappadocia to Queen Square. In *Background to Migraine*. ed (ED), S.R. London.

Cumberbatch, M.J., Hill, R.G. & Hargreaves, R.J. (1997). Rizatriptan has central antinociceptive effects against durally evoked responses. *Eur J Pharmacol*, **328,** 37-40.

Cutrer, F.M., O'Donnell, A. & Sanchez del Rio, M. (2000). Functional neuroimaging: enhanced understanding of migraine pathophysiology. *Neurology*, **55,** S36-45.

Cutrer, F.M., Yu, X.J., Ayata, G., Moskowitz, M.A. & Waeber, C. (1999). Effects of PNU-109,291, a selective 5-HT$_{1D}$ receptor agonist, on electrically induced dural plasma extravasation and capsaicin-evoked c-fos immunoreactivity within trigeminal nucleus caudalis. *Neuropharmacology*, **38,** 1043-1053.

Dahlöf, C.G.H. & Saxena, P.R. (2000). Novel compounds in development for acute treatment of migraine. In *The headaches*. eds OLESEN, J., TFELT-HANSEN, P. & WELCH, K.M.A. pp. 439-443. Philadelphia: Lippincott Wiliams & Wilkins.

Data, J., Britch, N. & Westergaard (1998). A double-blind study of ganaxolone in the acute treatment of migraine headaches with or without an aura in premenstrual females. *Headache*, **38,** 380.

de Hoon, J.N., Pickkers, P., Smits, P., Struijker-Boudier, H.A. & Van Bortel, L.M. (2003). Calcitonin gene-related peptide: exploring its vasodilating mechanism of action in humans. *Clin Pharmacol Ther*, **73,** 312-321.

De Vries, P., Heiligers, J.P., Villalón, C.M. & Saxena, P.R. (1996a). Blockade of porcine carotid vascular response to sumatriptan by GR 127935, a selective 5-HT1D receptor antagonist. *Br J Pharmacol*, **118,** 85-92.

De Vries, P., Heiligers, J.P.C., Villalón, C.M. & Saxena, P.R. (1996b). Blockade of porcine carotid vascular response to sumatriptan by GR127935, a selective 5-HT$_{1D}$ receptor antagonist. *Br. J. Pharmacol.*, **118,** 85-92.

De Vries, P., Sanchez-Lopez, A., Centurión, D., Heiligers, J.P., Saxena, P.R. & Villalón, C.M. (1998). The canine external carotid vasoconstrictor 5-HT1 receptor: blockade by 5-HT$_{1B}$ (SB224289), but not by 5-HT$_{1D}$ (BRL15572) receptor antagonists. *Eur J Pharmacol*, **362,** 69-72.

De Vries, P., Villalón, C.M. & Saxena, P.R. (1999a). Pharmacological aspects of experimental headache models in relation to acute antimigraine therapy. *Eur.J.Pharmacol.*, **375,** 61-74.

De Vries, P., Villalón, C.M. & Saxena, P.R. (1999b). Pharmacology of tryptans. *Emerging Drugs*, **4,** 107-125.

De Vries, P., Willems, E.W., Heiligers, J.P., Villalón, C.M. & Saxena, P.R. (1999c). Investigation of the role of 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors in the sumatriptan-induced constriction of porcine carotid arteriovenous anastomoses. *Br J Pharmacol*, **127,** 405-412.

Den Boer, M.O., Somers, J.A. & Saxena, P.R. (1992). Comparative effects of the antimigraine drugs sumatriptan and ergotamine on the distribution of cardiac output in anaesthetized pigs. *Cephalalgia*, **12,** 206-213.

Den Boer, M.O., Van Woerkens, L.J., Somers, J.A., Duncker, D.J., Lachmann, B., Saxena, P.R. & Verdouw, P.D. (1993). On the preservation and regulation of vascular tone in arteriovenous anastomoses during anesthesia. *J. Appl. Physiol.*, **75,** 782-789.

Diener, H.C., Hartung, J.A. & Gobel. (1995). Substance-P antagonist RPR 100893-201 is not effective in human migraine attacks. In *In 6th International Headache Research seminar*. Copenhagen.

Dingemanse, J., Gray, J.A., Soubrouillard, C., Jouve, E., Chaveau, J. & Blin, O. (1999). Pilot efficacy study of IS-159, a peptide serotonin $_{1B/1D}$ receptor agonist, after intranasal application in migraineurs. In *9th Congress of the International Headache Society*. Barcelona, Spain.

DiPette, D.J., Schwarzenberger, K., Kerr, N. & Holland, O.B. (1989). Dose-dependent systemic and regional haemodynamic effects of calcitonin gene-related peptide. *Am. J. Med. Sci.*, **297,** 65-70.

Doggrell, S.A. (2001). Migraine and beyond: cardiovascular therapeutic potential for CGRP modulators. *Expert Opin Investig Drugs*, **10,** 1131-1138.

Donaldson, C., Boers, P.M., Hoskin, K.L., Zagami, A.S. & Lambert, G.A. (2002). The role of 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors in the selective inhibitory effect of naratriptan on trigeminovascular neurons. *Neuropharmacology*, **42,** 374-385.

Doods, H. (2001). Development of CGRP antagonists for the treatment of migraine. *Curr. Opin. Investig. Drugs*, **2,** 1261-1268.

Doods, H., Hallermayer, G., Wu, D., Entzeroth, M., Rudolf, K., Engel, W. & Eberlein, W. (2000). Pharmacological profile of BIBN4096BS, the first selective small molecule CGRP antagonist. *Br. J. Pharmacol.*, **129,** 420-423.

Dray, A. (1992a). Neuropharmacological mechanisms of capsaicin and related substances. *Biochem Pharmacol*, **44,** 611-615.

Dray, A. (1992b). Mechanism of action of capsaicin-like molecules on sensory neurons. *Life Sci*, **51,** 1759-1765.

Drummond, P.D. & Lance, J.W. (1983). Extracranial vascular changes and the source of pain in migraine headache. *Ann Neurol*, **13,** 32-37.

Drummond, P.D. & Lance, J.W. (1984). Facial temperature in migraine, tension-vascular and tension headache. *Cephalalgia*, **4,** 149-158.

Du, X.Y., Schoemaker, R.G., Bos, E. & Saxena, P.R. (1994). Different pharmacological responses of atrium and ventricle: studies with human cardiac tissue. *Eur. J. Pharmacol.*, **259,** 173-180.

Durham, P. & Russo, A. (2002). New insights into the molecular actions of serotoninergic antimigraine drugs. *Pharmacol Ther*, **94,** 77-92.

Dwenger, A. (1984). Radioimmunoassay: an overview. *J. Clin. Chem. Clin. Biochem.*, **22,** 883-894.

Ebersberger, A., Averbeck, B., Messlinger, K. & Reeh, P.W. (1999). Release of substance P, calcitonin gene-related peptide and prostaglandin E2 from rat dura mater encephali following electrical and chemical stimulation in vitro. *Neuroscience*, **89,** 901-907.

Edmeads, J. (1991). What is migraine? Controversy and stalemate in migraine pathophysiology. *J Neurol*, **238 Suppl 1,** S2-5.

Edvinsson, L. (2001a). Sensory nerves in man and their role in primary headaches. *Cephalalgia*, **21,** 761-764.

Edvinsson, L. (2001b). Calcitonin gene-related peptide (CGRP) and the pathophysiology of headache: therapeutic implications. *CNS Drugs*, **15,** 745-753.

Edvinsson, L. (2002). Calcitonin gene-related Peptide (CGRP) in cerebrovascular disease. *Scientific World Journal*, **2,** 1484-1490.

Edvinsson, L. (2003). New therapeutic target in primary headaches - blocking the CGRP receptor. *Expert Opin Ther Targets*, **7,** 377-383.

Edvinsson, L., Alm, R., Shaw, D., Rutledge, R.Z., Koblan, K.S., Longmore, J. & Kane, S.A. (2002). Effect of the CGRP receptor antagonist BIBN4096BS in human cerebral, coronary and omental arteries and in SK-N-MC cells. *Eur J Pharmacol*, **434,** 49-53.

Edvinsson, L., Sams, A., Jansen-Olesen, I., Tajti, J., Kane, S.A., Rutledge, R.Z., Koblan, K.S., Hill, R.G. & Longmore, J. (2001). Characterisation of the effects of a non-peptide CGRP receptor antagonist in SK-N-MC cells and isolated human cerebral arteries. *Eur J Pharmacol*, **415,** 39-44.

Eglen, R.M., Jasper, J.R., Chang, D.A. & Martin, G.R. (1997). The 5-HT$_7$ receptor: orphan found. *Trends Pharmacol. Sci.*, **18,** 104-107.

Elkind, A.H. & Friedman, A.P. (1962). A review of headache: 1955 to 1961. III. *N Y State J Med*, **62,** 1649-1654.

Ellington, H.C., Cotter, M.A., Cameron, N.E. & Ross, R.A. (2002). The effect of cannabinoids on capsaicin-evoked calcitonin gene-related peptide (CGRP) release from the isolated paw skin of diabetic and non-diabetic rats. *Neuropharmacology*, **42,** 966-975.

Eltorp, C.T., Jansen-Olesen, I. & Hansen, A.J. (2000). Release of calcitonin gene-related peptide (CGRP) from guinea pig dura mater in vitro is inhibited by sumatriptan but unaffected by nitric oxide. *Cephalalgia*, **20,** 838-844.

Ennis, M.D., Ghazal, N.B., Hoffman, R.L., Smith, M.W., Schlachter, S.K., Lawson, C.F., Im, W.B., Pregenzer, J.F., Svensson, K.A., Lewis, R.A., Hall, E.D., Sutter, D.M., Harris, L.T. & McCall, R.B. (1998). Isochroman-6-carboxamides as highly selective 5-HT$_{1D}$ agonists: potential new treatment for migraine without cardiovascular side effects. *J Med Chem*, **41,** 2180-2183.

Escott, K.J. & Brain, S.D. (1993). Effect of a calcitonin gene-related peptide antagonist (CGRP8-37) on skin vasodilatation and oedema induced by stimulation of the rat saphenous nerve. *Br J Pharmacol*, **110,** 772-776.

Evans, B.N., Rosenblatt, M.I., Mnayer, L.O., Oliver, K.R. & Dickerson, I.M. (2000). CGRP-RCP, a novel protein required for signal transduction at calcitonin gene-related peptide and adrenomedullin receptors. *J Biol Chem*, **275,** 31438-31443.

Feniuk, W., Humphrey, P.P., Perren, M.J., Connor, H.E. & Whalley, E.T. (1991). Rationale for the use of 5-HT1-like agonists in the treatment of migraine. *J. Neurol.*, **238,** S57-61.

Ferrari, M.D. (1998). Migraine. *Lancet*, **351,** 1043-1051.

Ferrari, M.D. & Saxena, P.R. (1993a). On serotonin and migraine: a clinical and pharmacological review. *Cephalalgia*, **13,** 151-165.

Ferrari, M.D. & Saxena, P.R. (1993b). Clinical and experimental effects of sumatriptan in humans. *Trends Pharmacol Sci*, **14,** 129-133.

Fitzsimmons, T.J., Zhao, X. & Wank, S.A. (2003). The extracellular domain of receptor activity-modifying protein 1 is sufficient for calcitonin receptor-like receptor function. *J Biol Chem*, **278,** 14313-14320.

Foord, S.M., Wise, A., Brown, J., Main, M.J. & Fraser, N.J. (1999). The N-terminus of RAMPs is a critical determinant of the glycosylation state and ligand binding of calcitonin receptor-like receptor. *Biochem Soc Trans*, **27,** 535-539.

Franco-Cereceda, A., Bengtsson, L. & Lundberg, J.M. (1987). Inotropic effects of calcitonin gene-related peptide, vasoactive intestinal polypeptide and somatostatin on the human right atrium in vitro. *Eur J Pharmacol*, **134,** 69-76.

Fusco, M., D'Andrea, G., Micciche, F., Stecca, A., Bernardini, D. & Cananzi, A.L. (2003). Neurogenic inflammation in primary headaches. *Neurol Sci*, **24 Suppl 2,** S61-64.

Gangula, P.R., Dong, Y.L., Wimalawansa, S.J. & Yallampalli, C. (2002). Infusion of pregnant rats with calcitonin gene-related peptide (CGRP)(8-37), a CGRP receptor antagonist, increases blood pressure and fetal mortality and decreases fetal growth. *Biol Reprod*, **67,** 624-629.

Gardiner, S.M., Compton, A.M., Kemp, P.A., Bennett, T., Bose, C., Foulkes, R. & Hughes, B. (1990). Antagonistic effect of human α-CGRP$_{8-37}$ on the in vivo regional haemodynamic actions of human α-CGRP. *Biochem. Biophys. Res. Commun.*, **171,** 938-943.

Gennari, C. & Fischer, J.A. (1985). Cardiovascular action of calcitonin gene-related peptide in humans. *Calcif Tissue Int*, **37,** 581-584.

Gibson, S.J., Polak, J.M., Giaid, A., Hamid, Q.A., Kar, S., Jones, P.M., Denny, P., Legon, S., Amara, S.G., Craig, R.K. & et al. (1988). Calcitonin gene-related peptide messenger RNA is expressed in sensory neurones of the dorsal root ganglia and also in spinal motorneurones in man and rat. *Neurosci Lett*, **91,** 283-288.

Giffin, N.J., Kowacs, F., Libri, V., Williams, P., Goadsby, P.J. & Kaube, H. (2003). Effect of the adenosine A1 receptor agonist GR79236 on trigeminal nociception with blink reflex recordings in healthy human subjects. *Cephalalgia*, **23,** 287-292.

Girgis, S.I., Macdonald, D.W., Stevenson, J.C., Bevis, P.J., Lynch, C., Wimalawansa, S.J., Self, C.H., Morris, H.R. & MacIntyre, I. (1985). Calcitonin gene-related peptide: potent vasodilator and major product of calcitonin gene. *Lancet*, **2,** 14-16.

Goadsby, P.J. (1993). Inhibition of calcitonin gene-related peptide by h-CGRP$_{8-37}$ antagonizes the cerebral dilator response from nasociliary nerve stimulation in the cat. *Neurosci. Lett.*, **151,** 13-16.

Goadsby, P.J. (1997a). Is a central action of acute antimigraine drugs essential? *Cephalalgia*, **17 Suppl 17,** 10-11.

Goadsby, P.J. (1997b). Current concepts of the pathophysiology of migraine. *Neurol. Clin.*, **15,** 27-42.

Goadsby, P.J. (1998). Serotonin 5-HT$_{1B/1D}$ receptor agonists in migraine. Comparative pharmacology and its therapeutic implications. *CNS Drugs*, **10,** 271-286.

Goadsby, P.J. (1999). Advances in the pharmacotherapy of migraine. How knowledge of pathophysiology is guiding drug development. *Drug Res. Dev.*, **2,** 361-374.

Goadsby, P.J. (2000). The pharmacology of headache. *Prog Neurobiol*, **62,** 509-525.

Goadsby, P.J. & Boes, C.J. (2001). Zolmitriptan: differences from sumatriptan. *Curr Med Res Opin*, **17 Suppl 1,** s46-50.

Goadsby, P.J. & Edvinsson, L. (1993). The trigeminovascular system and migraine: studies characterizing cerebrovascular and neuropeptide changes seen in humans and cats. *Ann Neurol*, **33,** 48-56.

Goadsby, P.J., Edvinsson, L. & Ekman, R. (1988). Release of vasoactive peptides in the extracerebral circulation of humans and the cat during activation of the trigeminovascular system. *Ann Neurol*, **23,** 193-196.

Goadsby, P.J., Edvinsson, L. & Ekman, R. (1990). Vasoactive peptide release in the extracerebral circulation of humans during migraine headache. *Ann Neurol*, **28,** 183-187.

Goadsby, P.J. & Hoskin, K.L. (1997). The distribution of trigeminovascular afferents in the nonhuman primate brain Macaca nemestrina: a c-fos immunocytochemical study. *J Anat*, **190 (Pt 3),** 367-375.

Goadsby, P.J. & Hoskin, K.L. (1999). Differential effects of low dose CP122,288 and eletriptan on fos expression due to stimulation of the superior sagittal sinus in cat. *Pain*, **82,** 15-22.

Goadsby, P.J., Hoskin, K.L., Storer, R.J., Edvinsson, L. & Connor, H.E. (2002a). Adenosine A1 receptor agonists inhibit trigeminovascular nociceptive transmission. *Brain*, **125,** 1392-1401.

Goadsby, P.J. & Knight, Y.E. (1997). Direct evidence for central sites of action of zolmitriptan (311C90): an autoradiographic study in cat [In Process Citation]. *Cephalalgia*, **17,** 153-158; discussion 143.

Goadsby, P.J., Lipton, R.B. & Ferrari, M.D. (2002b). Migraine--current understanding and treatment. *N Engl J Med*, **346,** 257-270.

Goldstein, D.J., Offen, W.W., Klein, E.G., Phebus, L.A., Hipskind, P., Johnson, K.W. & Ryan, R.E., Jr. (2001a). Lanepitant, an NK-1 antagonist, in migraine prevention. *Cephalalgia*, **21,** 102-106.

Goldstein, D.J., Roon, K.I., Offen, W.W., Ramadan, N.M., Phebus, L.A., Johnson, K.W., Schaus, J.M. & Ferrari, M.D. (2001b). Selective serotonin 1F (5-ht(1F)) receptor agonist LY334370 for acute migraine: a randomised controlled trial. *Lancet*, **358,** 1230-1234.

Goldstein, D.J., Wang, O., Saper, J.R., Stoltz, R., Silberstein, S.D. & Mathew, N.T. (1997). Ineffectiveness of neurokinin-1 antagonist in acute migraine: a crossover study. *Cephalalgia*, **17,** 785-790.

Goldstein, J., Dahlof, C.G., Diener, H.C., Olesen, J., Schellens, R., Senard, J.M., Simard, D. & Steiner, T.J. (1996). Alniditan in the acute treatment of migraine attacks: a subcutaneous dose-finding study. Subcutaneous Alniditan Study Group. *Cephalalgia*, **16,** 497-502.

Gómez-Mancilla, B., Cutler, N.R., Leibowitz, M.T., Spierings, E.L., Klapper, J.A., Diamond, S., Goldstein, J., Smith, T., Couch, J.R., Fleishaker, J., Azie, N. & Blunt, D.E. (2001). Safety and efficacy of PNU-142633, a selective 5-HT$_{1D}$ agonist, in patients with acute migraine. *Cephalalgia*, **21,** 727-732.

Gulbenkian, S., Barroso, C.P., Cunha e Sa, M. & Edvinsson, L. (1995). The peptidergic innervation of human coronary and cerebral vessels. *Ital. J. Anat. Embryol.*, **100,** 317-327.

Gulbenkian, S., Uddman, R. & Edvinsson, L. (2001). Neuronal messengers in the human cerebral circulation. *Peptides*, **22,** 995-1007.

Hagan, J.J., Slade, P.D., Gaster, L., Jeffrey, P., Hatcher, J.P. & Middlemiss, D.N. (1997). Stimulation of 5-HT$_{1B}$ receptors causes hypothermia in the guinea pig. *Eur. J. Pharmacol.*, **331,** 169-174.

Hagner, S., Haberberger, R.V., Overkamp, D., Hoffmann, R., Voigt, K.H. & McGregor, G.P. (2002a). Expression and distribution of calcitonin receptor-like receptor in human hairy skin. *Peptides*, **23,** 109-116.

Hagner, S., Knauer, J., Haberberger, R., Goke, B., Voigt, K. & McGregor, G.P. (2002b). Calcitonin receptor-like receptor is expressed on gastrointestinal immune cells. *Digestion*, **66,** 197-203.

Hagner, S., Stahl, U., Knoblauch, B., McGregor, G.P. & Lang, R.E. (2002c). Calcitonin receptor-like receptor: identification and distribution in human peripheral tissues. *Cell Tissue Res*, **310,** 41-50.

Hales, J. (1974). Radioactive microsphere techniques for studies of the circulation. *Clin. exp. Pharmacol. Physiol.*, **1,** 31-46.

Hamalainen, M.L., Autti, T., Salonen, O. & Santavuori, P. (1996). Brain MRI in children with migraine: a controlled morphometric study. *Cephalalgia*, **16,** 541-544.

Hargreaves, R.J., Williamson, D.J. & Shepheard, S.L. (1999). Neurogenic inflammation: relation to novel antimigraine drugs. In *Migraine and headache pathophysiology*. ed EDVINSSON, L. pp. 93-101. London: Martin Dunitz.

Harper, A.M., MacKenzie, E.T., McCulloch, J. & Pickard, J.D. (1977). Migraine and the blood-brain barrier. *Lancet*, **1,** 1034-1036.

Hasbak, P., Opgaard, O.S., Eskesen, K., Schifter, S., Arendrup, H., Longmore, J. & Edvinsson, L. (2003). Investigation of CGRP receptors and peptide pharmacology in human coronary arteries. Characterization with a nonpeptide antagonist. *J Pharmacol Exp Ther*, **304,** 326-333.

Hasbak, P., Sams, A., Schifter, S., Longmore, J. & Edvinsson, L. (2001). CGRP receptors mediating CGRP-, adrenomedullin- and amylin-induced relaxation in porcine coronary arteries. Characterization with 'Compound 1' (WO98/11128), a non-peptide antagonist. *Br. J. Pharmacol.*, **133,** 1405-1413.

Hauser, W.A., Annegers, J.F. & Kurland, L.T. (1991). Prevalence of epilepsy in Rochester, Minnesota: 1940-1980. *Epilepsia*, **32,** 429-445.

Hay, D.L., Howitt, S.G., Conner, A.C., Doods, H., Schindler, M. & Poyner, D.R. (2002). A comparison of the actions of BIBN4096BS and CGRP(8-37) on CGRP and adrenomedullin receptors expressed on SK-N-MC, L6, Col 29 and Rat 2 cells. *Br J Pharmacol*, **137,** 80-86.

Heyck, H. (1969). Pathogenesis of migraine. *Res. Clin. Stud. Headache*, **2,** 1-28.

Hibbs, J.B., Jr., Taintor, R.R., Vavrin, Z. & Rachlin, E.M. (1988). Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem Biophys Res Commun*, **157,** 87-94.

Hilairet, S., Foord, S.M., Marshall, F.H. & Bouvier, M. (2001). Protein-protein interaction and not glycosylation determines the binding selectivity of heterodimers between the calcitonin receptor-like receptor and the receptor activity-modifying proteins. *J Biol Chem*, **276,** 29575-29581.

Hoffmann, O., Keilwerth, N., Bille, M.B., Reuter, U., Angstwurm, K., Schumann, R.R., Dirnagl, U. & Weber, J.R. (2002). Triptans reduce the inflammatory response in bacterial meningitis. *J Cereb Blood Flow Metab*, **22,** 988-996.

Hoppener, J.W., Steenbergh, P.H., Zandberg, J., Geurts van Kessel, A.H., Baylin, S.B., Nelkin, B.D., Jansz, H.S. & Lips, C.J. (1985). The second human calcitonin/CGRP gene is located on chromosome 11. *Hum Genet*, **70,** 259-263.

Hou, M., Uddman, R., Tajti, J., Kanje, M. & Edvinsson, L. (2002). Capsaicin receptor immunoreactivity in the human trigeminal ganglion. *Neurosci. Lett.*, **330,** 223-226.

Howitt, S.G. & Poyner, D.R. (1997). The selectivity and structural determinants of peptide antagonists at the CGRP receptor of rat, L6 myocytes. *Br J Pharmacol*, **121,** 1000-1004.

Hoyer, D., Clarke, D.E., Fozard, J.R., Hartig, P.R., Martin, G.R., Mylecharane, E.J., Saxena, P.R. & Humphrey, P.P. (1994). International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). *Pharmacol. Rev.*, **46,** 157-203.

Humphrey, P.P. & Feniuk, W. (1991). Mode of action of the anti-migraine drug sumatriptan. *Trends Pharmacol Sci*, **12,** 444-446.

Humphrey, P.P., Feniuk, W., Perren, M.J., Connor, H.E. & Oxford, A.W. (1989). The pharmacology of the novel 5-HT1-like receptor agonist, GR43175. *Cephalalgia*, **9 Suppl 9,** 23-33.

Irie, K., Hara-Irie, F., Ozawa, H. & Yajima, T. (2002). Calcitonin gene-related peptide (CGRP)-containing nerve fibers in bone tissue and their involvement in bone remodeling. *Microsc Res Tech*, **58,** 85-90.

Ishikawa, T., Okamura, N., Saito, A. & Goto, K. (1987). Effects of calcitonin gene-related peptide (CGRP) and isoproterenol on the contractility and adenylate cyclase activity in the rat heart. *J. Mol. Cell Cardiol.*, **19,** 723-727.

Jansen, I., Alafaci, C., Uddman, R. & Edvinsson, L. (1990). Evidence that calcitonin gene-related peptide contributes to the capsaicin-induced relaxation of guinea pig cerebral arteries. *Regul. Pept.*, **31,** 167-178.

Jansen-Olesen, I., Mortensen, A. & Edvinsson, L. (1996). Calcitonin gene-related peptide is released from capsaicin-sensitive nerve fibres and induces vasodilatation of human cerebral arteries concomitant with activation of adenylyl cyclase. *Cephalalgia*, **16,** 310-316.

Jarvikallio, A., Harvima, I.T. & Naukkarinen, A. (2003). Mast cells, nerves and neuropeptides in atopic dermatitis and nummular eczema. *Arch Dermatol Res*, **295,** 2-7.

Johnson, K.W., Schaus, J.M., Durkin, M.M., Audia, J.E., Kaldor, S.W., Flaugh, M.E., Adham, N., Zgombick, J.M., Cohen, M.L., Branchek, T.A. & Phebus, L.A. (1997). 5-ht$_{1F}$ receptor agonists inhibit neurogenic dural inflammation in guinea pigs. *Neuroreport*, **8,** 2237-2240.

Jonas, V., Lin, C.R., Kawashima, E., Semon, D., Swanson, L.W., Mermod, J.J., Evans, R.M. & Rosenfeld, M.G. (1985). Alternative RNA processing events in human calcitonin/calcitonin gene-related peptide gene expression. *Proc Natl Acad Sci U S A*, **82,** 1994-1998.

Juaneda, C., Dumont, Y. & Quirion, R. (2000). The molecular pharmacology of CGRP and related peptide receptor subtypes. *Trends Pharmacol Sci*, **21,** 432-438.

Kallner, G., Gonon, A. & Franco-Cereceda, A. (1998). Calcitonin gene-related peptide in myocardial ischemia and reperfusion in the pig. *Cardiovasc. Res.*, **38,** 493-499.

Kangrga, I., Larew, J.S. & Randic, M. (1990). The effects of substance P and calcitonin gene-related peptide on the efflux of endogenous glutamate and aspartate from the rat spinal dorsal horn *in vitro*. *Neurosci Lett*, **108,** 155-160.

Kapicioglu, S., Gokce, E., Kapicioglu, Z. & Ovali, E. (1997). Treatment of migraine attacks with a long-acting somatostatin analogue (octreotide, SMS 201-995). *Cephalalgia*, **17,** 27-30.

Kapoor, K., Arulmani, U., Heiligers, J.P., Garrelds, I.M., Willems, E.W., Doods, H., Villalon, C.M. & Saxena, P.R. (2003a). Effects of the CGRP receptor antagonist BIBN4096BS on capsaicin-induced carotid haemodynamic changes in anaesthetised pigs. *Br J Pharmacol*, **140,** 329-338.

Kapoor, K., Arulmani, U., Heiligers, J.P., Willems, E.W., Doods, H., Villalon, C.M. & Saxena, P.R. (2003b). Effects of BIBN4096BS on cardiac output distribution and on CGRP-induced carotid haemodynamic responses in the pig. *Eur J Pharmacol*, **475,** 69-77.

Kapoor, K., Heiligers, J.P.C., Willems, E.W. & Saxena, P.R. (2002). Effects of BIBN4096BS - a novel CGRP antagonist on the capsaicin-induced haemodynamic changes in anaesthetized pigs. *Pharmacologist*, **44 (Suppl. 1),** A151.

Kawasaki, H., Inaizumi, K., Nakamura, A., Hobara, N. & Kurosaki, Y. (2003). Chronic angiotensin II inhibition increases levels of calcitonin gene-related peptide *mRNA* of the dorsal root ganglia in spontaneously hypertensive rats. *Hypertens Res*, **26,** 257-263.

Kawasaki, H., Nuki, C., Saito, A. & Takasaki, K. (1990). Role of calcitonin gene-related peptide-containing nerves in the vascular adrenergic neurotransmission. *J. Pharmacol. Exp. Ther.*, **252,** 403-409.

Kaygisiz, Z., Erksap, N., Uyar, R., Kabadere, S., Kabadere, T.E. & Dernek, S. (2003). The effect of adrenomedullin, amylin fragment8-37 and calcitonin gene-related peptide on contractile force, heart rate and coronary perfusion pressure in isolated rat hearts. *Acta Physiol Hung*, **90,** 133-146.

Khasar, S.G., Green, P.G., Chou, B. & Levine, J.D. (1995). Peripheral nociceptive effects of alpha 2-adrenergic receptor agonists in the rat. *Neuroscience*, **66,** 427-432.

Kitamura, K., Kangawa, K., Kawamoto, M., Ichiki, Y., Nakamura, S., Matsuo, H. & Eto, T. (1993). Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem Biophys Res Commun*, **192,** 553-560.

Knerr, I., Dachert, C., Beinder, E., Metzler, M., Dotsch, J., Repp, R. & Rascher, W. (2002). Adrenomedullin, calcitonin gene-related peptide and their receptors: evidence for a decreased placental *mRNA* content in preeclampsia and HELLP syndrome. *Eur J Obstet Gynecol Reprod Biol*, **101,** 47-53.

Knight, Y.E., Edvinsson, L. & Goadsby, P.J. (1999). Blockade of calcitonin gene-related peptide release after superior sagittal sinus stimulation in cat: a comparison of avitriptan and CP122,288. *Neuropeptides*, **33,** 41-46.

Kolesnikov, Y.A., Pick, C.G. & Pasternak, G.W. (1992). NG-nitro-L-arginine prevents morphine tolerance. *Eur J Pharmacol*, **221,** 399-400.

Kuo, T., Ouchi, Y., Kim, S., Toba, K. & Orimo, H. (1994). The role of activation of the sympathetic nervous system in the central pressor action of calcitonin gene-related peptide in conscious rats. *Naunyn Schmiedebergs Arch Pharmacol*, **349,** 394-400.

Lambert, G. & Michalicek, J. (1996). Effect of antimigraine drugs on dural blood flows and resistances and the responses to trigeminal stimulation. *Eur J Pharmacol*, **311,** 141-151.

Lambert, G.A., Boers, P.M., Hoskin, K.L., Donaldson, C. & Zagami, A.S. (2002). Suppression by eletriptan of the activation of trigeminovascular sensory neurons by glyceryl trinitrate. *Brain Res*, **953,** 181-188.

Langer, S.Z. (1980). Presynaptic regulation of the release of catecholamines. *Pharmacol. Rev.*, **32,** 337-362.

Lassen, L.H., Ashina, M., Christiansen, I., Ulrich, V. & Olesen, J. (1997). Nitric oxide synthase inhibition in migraine. *Lancet*, **349,** 401-402.

Lassen, L.H., Haderslev, P.A., Jacobsen, V.B., Iversen, H.K., Sperling, B. & Olesen, J. (2002). CGRP may play a causative role in migraine. *Cephalalgia*, **22,** 54-61.

Li, Y.J. & Peng, J. (2002). The cardioprotection of calcitonin gene-related peptide-mediated preconditioning. *Eur J Pharmacol*, **442,** 173-177.

Limmroth, V., Katsarava, Z., Liedert, B., Guehring, H., Schmitz, K., Diener, H.C. & Michel, M.C. (2001). An *in vivo* rat model to study calcitonin gene related peptide release following activation of the trigeminal vascular system. *Pain*, **92,** 101-106.

Lincoln, T.M. (1989). Cyclic GMP and mechanisms of vasodilation. *Pharmacol Ther*, **41,** 479-502.

Lipton, R.B., Ottman, R., Ehrenberg, B.L. & Hauser, W.A. (1994). Comorbidity of migraine: the connection between migraine and epilepsy. *Neurology*, **44,** S28-32.

Longmore, J., Shaw, D., Smith, D., Hopkins, R., McAllister, G., Pickard, J.D., Sirinathsinghji, D.J., Butler, A.J. & Hill, R.G. (1997). Differential distribution of 5HT$_{1D}$- and 5HT$_{1B}$-immunoreactivity within the human trigemino-cerebrovascular system: implications for the discovery of new antimigraine drugs. *Cephalalgia*, **17,** 833-842.

Low, N.C. & Merikangas, K.R. (2003). The comorbidity of migraine. *CNS Spectr*, **8,** 433-434, 437-444.

Lu, R., Li, Y.J. & Deng, H.W. (1999). Evidence for calcitonin gene-related peptide-mediated ischemic preconditioning in the rat heart. *Regul. Pept.*, **82,** 53-57.

Ludbrook, J. (1994). Repeated measurements and multiple comparisons in cardiovascular research. *Cardiovasc. Res.*, **28,** 303-311.

Lundberg, J.M., Franco-Cereceda, A., Hua, X., Hokfelt, T. & Fischer, J.A. (1985). Co-existence of substance P and calcitonin gene-related peptide-like immunoreactivities in sensory nerves in relation to cardiovascular and bronchoconstrictor effects of capsaicin. *Eur J Pharmacol*, **108,** 315-319.

Ma, W., Chabot, J.G., Powell, K.J., Jhamandas, K., Dickerson, I.M. & Quirion, R. (2003). Localization and modulation of calcitonin gene-related peptide-receptor component protein-immunoreactive cells in the rat central and peripheral nervous systems. *Neuroscience*, **120,** 677-694.

Maassen VanDenBrink, A., Bax, W.A., Ramrattan, N.N., Ferrari, M.D. & Saxena, P.R. (1999). Human isolated coronary artery contraction to sumatriptan: a post hoc analysis. *Cephalalgia*, **19,** 651-654.

MaassenVanDenBrink, A., Reekers, M., Bax, W.A. & Saxena, P.R. (2000a). Human isolated coronary artery contraction to sumatriptan characterised by the selective 5-HT$_{1B/1D}$ receptor antagonist GR55562. *Pharmacol. Toxicol.*, **86,** 287-290.

MaassenVanDenBrink, A., van den Broek, R.W., de Vries, R., Bogers, A.J., Avezaat, C.J. & Saxena, P.R. (2000b). Craniovascular selectivity of eletriptan and sumatriptan in human isolated blood vessels. *Neurology*, **55,** 1524-1530.

Mallee, J.J., Salvatore, C.A., LeBourdelles, B., Oliver, K.R., Longmore, J., Koblan, K.S. & Kane, S.A. (2002). Receptor activity-modifying protein 1 determines the species selectivity of non-peptide CGRP receptor antagonists. *J Biol Chem*, **277,** 14294-14298.

Marshall, I. (1992). Mechanism of vascular relaxation by the calcitonin gene-related peptide. *Ann N Y Acad Sci*, **657,** 204-215.

Marshall, I., Al-Kazwini, S.J., Roberts, P.M., Shepperson, N.B., Adams, M. & Craig, R.K. (1986). Cardiovascular effects of human and rat CGRP compared in the rat and other species. *Eur J Pharmacol*, **123,** 207-216.

Mathew, N.T. (2001). Pathophysiology, epidemiology, and impact of migraine. *Clin Cornerstone*, **4,** 1-17.

May, A., Gijsman, H.J., Wallnofer, A., Jones, R., Diener, H.C. & Ferrari, M.D. (1996). Endothelin antagonist bosentan blocks neurogenic inflammation, but is not effective in aborting migraine attacks. *Pain*, **67,** 375-378.

May, A., Shepheard, S.L., Knorr, M., Effert, R., Wessing, A., Hargreaves, R.J., Goadsby, P.J. & Diener, H.C. (1998). Retinal plasma extravasation in animals but not in humans: implications for the pathophysiology of migraine. *Brain*, **121 (Pt 7),** 1231-1237.

McCall, R.B. (1999). Preclinical and clinical studies in migraine using the selective 5-HT$_{1D}$ receptor agonist PNU-142633. In *IBC's 3rd annual conference on migraine*. Philadelphia, USA.

McLatchie, L.M., Fraser, N.J., Main, M.J., Wise, A., Brown, J., Thompson, N., Solari, R., Lee, M.G. & Foord, S.M. (1998). RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature*, **393,** 333-339.

Medeiros, M.V., De Luca, I.M., Bricola, A.A., Zanesco, A., De Nucci, G. & Antunes, E. (2003). Enhanced airways responsiveness in rats depleted of sensory neuropeptides by neonatal capsaicin treatment. *Neurosci Lett*, **341,** 103-106.

Merikangas, K.R., Angst, J. & Isler, H. (1990). Migraine and psychopathology. Results of the Zurich cohort study of young adults. *Arch Gen Psychiatry*, **47,** 849-853.

Miyamoto, Y., Yoneda, M., Morikawa, A., Itoh, H. & Makino, I. (2001). Gastric neuropeptides and gastric motor abnormality in streptozotocin-induced diabetic rats: observation for four weeks after streptozotocin. *Dig Dis Sci*, **46,** 1596-1603.

Morara, S., Rosina, A., Provini, L., Forloni, G., Caretti, A. & Wimalawansa, S.J. (2000). Calcitonin gene-related peptide receptor expression in the neurons and glia of developing rat cerebellum: an autoradiographic and immunohistochemical analysis. *Neuroscience*, **100,** 381-391.

Morara, S., Wimalawansa, S.J. & Rosina, A. (1998). Monoclonal antibodies reveal expression of the CGRP receptor in Purkinje cells, interneurons and astrocytes of rat cerebellar cortex. *Neuroreport*, **9,** 3755-3759.

Moreno, M.J., Abounader, R., Hebert, E., Doods, H. & Hamel, E. (2002). Efficacy of the non-peptide CGRP receptor antagonist BIBN4096BS in blocking CGRP-induced dilations in human and bovine cerebral arteries: potential implications in acute migraine treatment. *Neuropharmacology*, **42,** 568-576.

Morris, H.R., Panico, M., Etienne, T., Tippins, J., Girgis, S.I. & MacIntyre, I. (1984). Isolation and characterization of human calcitonin gene-related peptide. *Nature*, **308,** 746-748.

Moskowitz, M.A. (1993). Neurogenic inflammation in the pathophysiology and treatment of migraine. *Neurology*, **43,** S16-20.

Moskowitz, M.A. & Buzzi, M.G. (1991). Neuroeffector functions of sensory fibres: implications for headache mechanisms and drug actions. *J. Neurol.*, **238,** S18-22.

Moskowitz, M.A., Buzzi, M.G., Sakas, D.E. & Linnik, M.D. (1989). Pain mechanisms underlying vascular headaches. Progress Report 1989. *Rev Neurol (Paris)*, **145,** 181-193.

Mulderry, P.K., Ghatei, M.A., Spokes, R.A., Jones, P.M., Pierson, A.M., Hamid, Q.A., Kanse, S., Amara, S.G., Burrin, J.M., Legon, S. & et al. (1988). Differential expression of alpha-CGRP and beta-CGRP by primary sensory neurons and enteric autonomic neurons of the rat. *Neuroscience*, **25,** 195-205.

Oh-hashi, Y., Shindo, T., Kurihara, Y., Imai, T., Wang, Y., Morita, H., Imai, Y., Kayaba, Y., Nishimatsu, H., Suematsu, Y., Hirata, Y., Yazaki, Y., Nagai, R., Kuwaki, T. & Kurihara, H. (2001). Elevated sympathetic nervous activity in mice deficient in alpha CGRP. *Circ Res*, **89,** 983-990.

Ohtori, S., Takahashi, K., Chiba, T., Yamagata, M., Sameda, H. & Moriya, H. (2002). Substance P and calcitonin gene-related peptide immunoreactive sensory DRG neurons innervating the lumbar intervertebral discs in rats. *Ann Anat*, **184,** 235-240.

Okimura, Y., Chihara, K., Abe, H., Kita, T., Kashio, Y., Sato, M. & Fujita, T. (1987). Calcitonin gene-related peptide-like immunoreactivity in the central nervous system and peripheral organs of rats. *Regul. Pept.*, **17,** 327-337.

Oku, R., Satoh, M., Fujii, N., Otaka, A., Yajima, H. & Takagi, H. (1987). Calcitonin gene-related peptide promotes mechanical nociception by potentiating release of substance P from the spinal dorsal horn in rats. *Brain Res*, **403,** 350-354.

Olesen, J. (1991a). Cerebral and extracranial circulatory disturbances in migraine: pathophysiological implications. *Cerebrovasc Brain Metab Rev*, **3,** 1-28.

Olesen, J. (1991b). A review of current drugs for migraine. *J Neurol*, **238 Suppl 1,** S23-27.

Olesen, J., Diener, H., Husstedt, I.W., Goadsby, P.J., David, H., Meier, U., Pollentier, S. & Lesko, L.M. (2003a). Calcitonin gene-realted peptide (CGRP) receptor antagonist BIBN4096BS is effective in the treatment of migraine attacks (Abstract of the XI congress of the International Headache Society/IHC 2003, Rome, Italy). *Cephalalgia*, **23,** 579.

Olesen, J., Goadsby, P. & Steiner, T. (2003b). The International Classification of Headache Disorders: 2nd edition. *Lancet Neurol*, **2,** 720.

Olesen, J. & Lassen, L.H. (2001). CGRP as a migraine inducing substance. *Scientific World Journal*, **1,** 31.

Olesen, J., Thomsen, L.L. & Iversen, H. (1994). Nitric oxide is a key molecule in migraine and other vascular headaches. *Trends Pharmacol Sci*, **15,** 149-153.

Ophoff, R.A., Terwindt, G.M., Vergouwe, M.N., van Eijk, R., Oefner, P.J., Hoffman, S.M., Lamerdin, J.E., Mohrenweiser, H.W., Bulman, D.E., Ferrari, M., Haan, J., Lindhout, D., van Ommen, G.J., Hofker, M.H., Ferrari, M.D. & Frants, R.R. (1996). Familial hemiplegic migraine and episodic ataxia type-2 are caused by mutations in the Ca2+ channel gene CACNL1A4. *Cell*, **87,** 543-552.

O'Shaughnessy, C.T. & Connor, H.E. (1994). Investigation of the role of tachykinin NK1, NK2 receptors and CGRP receptors in neurogenic plasma protein extravasation in dura mater. *Eur J Pharmacol*, **263,** 193-198.

O'Shaughnessy, C.T., Waldron, G.J. & Connor, H.E. (1993). Lack of effect of sumatriptan and UK-14,304 on capsaicin-induced relaxation of guinea-pig isolated basilar artery. *Br. J. Pharmacol.*, **108,** 191-195.

Overall, J.E. & Atlas, R.S. (1999). Power of univariate and multivariate analyses of repeated measurements in controlled clinical trials. *J. Clin. Psychol.*, **55,** 465-485.

Overall, J.E. & Doyle, S.R. (1994). Implications of chance baseline differences in repeated measurement designs. *J. Biopharm. Stat.*, **4,** 199-216.

Overall, J.E. & Doyle, S.R. (1996). False-positive error rates in routine application of repeated measurements ANOVA. *J. Biopharm. Stat.*, **6,** 69-81.

Peng, J., Xiao, J., Ye, F., Deng, H.W. & Li, Y.J. (2000). Inhibition of cardiac tumor necrosis factor-alpha production by calcitonin gene-related peptide-mediated ischemic preconditioning in isolated rat hearts. *Eur. J. Pharmacol.*, **407,** 303-308.

Peroutka, S.J. (1995). Drugs effective in the therapy of migraine. In *Goodman & Gilman's The pharmacological basis of therapeutics*. eds HARDMAN, J.G. & LIMBIRD, L.E. pp. 487-502. New York: McGraw-Hill.

Petzold, G.C. (2003). Migraine-induced stroke in a patient with migraine-related epilepsy. *Headache*, **43,** 694-696.

Phebus, L.A., Johnson, K.W., Zgombick, J.M., Gilbert, P.J., Van Belle, K., Mancuso, V., Nelson, D.L., Calligaro, D.O., Kiefer, A.D., Jr., Branchek, T.A. & Flaugh, M.E. (1997). Characterization of LY344864 as a pharmacological tool to study 5-ht$_{1F}$ receptors: binding affinities, brain penetration and activity in the neurogenic dural inflammation model of migraine. *Life Sci*, **61,** 2117-2126.

Poyner, D. & Marshall, I. (2001). CGRP receptors: beyond the CGRP$_1$-CGRP$_2$ subdivision? *Trends Pharmacol. Sci.*, **22,** 223.

Poyner, D.R., Sexton, P.M., Marshall, I., Smith, D.M., Quirion, R., Born, W., Muff, R., Fischer, J.A. & Foord, S.M. (2002). International Union of Pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. *Pharmacol Rev*, **54,** 233-246.

Prado, M.A., Evans-Bain, B. & Dickerson, I.M. (2002). Receptor component protein (RCP): a member of a multi-protein complex required for G-protein-coupled signal transduction. *Biochem Soc Trans*, **30,** 460-464.

Proudfoot, N.J. & Brownlee, G.G. (1976). 3' non-coding region sequences in eukaryotic messenger RNA. *Nature*, **263,** 211-214.

Qing, X. & Keith, I.M. (2003). Targeted blocking of gene expression for CGRP receptors elevates pulmonary artery pressure in hypoxic rats. *Am J Physiol Lung Cell Mol Physiol*, **285,** L86-96.

Ramadan, N.M. (2001). Acute treatments: some blind alleys. *Curr Med Res Opin*, **17 Suppl 1,** s71-80.

Ramadan, N.M., Skljarevski, V., Phebus, L.A. & Johnson, K.W. (2003). 5-HT1F receptor agonists in acute migraine treatment: a hypothesis. *Cephalalgia*, **23,** 776-785.

Rapoport, A. & Edmeads, J. (2000). Migraine: the evolution of our knowledge. *Arch Neurol*, **57,** 1221-1223.

Rasmussen, B.K., Jensen, R., Schroll, M. & Olesen, J. (1991). Epidemiology of headache in a general population--a prevalence study. *J Clin Epidemiol*, **44,** 1147-1157.

Rasmussen, T.N., Schmidt, P., Poulsen, S.S. & Holst, J.J. (2001). Localisation and neural control of the release of calcitonin gene- related peptide (CGRP) from the isolated perfused porcine ileum. *Regul. Pept.*, **98,** 137-143.

Raval, P., Bingham, S., Aiyar, N., Elliott, J.D., Hunter, A.J., Ohlstein, E.H. & Parsons, A.A. (1999). Trigeminal nerve ganglion stimulation-induced neurovascular reflexes in the anaesthetized cat: role of endothelin (B) receptors in carotid vasodilatation. *Br J Pharmacol*, **126,** 485-493.

Rist, B., Entzeroth, M. & Beck-Sickinger, A.G. (1998). From micromolar to nanomolar affinity: a systematic approach to identify the binding site of CGRP at the human calcitonin gene-related peptide 1 receptor. *J Med Chem*, **41,** 117-123.

Roa, M. & Changeux, J.P. (1991). Characterization and developmental evolution of a high-affinity binding site for calcitonin gene-related peptide on chick skeletal muscle membrane. *Neuroscience*, **41,** 563-570.

Roon, K.I., Olesen, J., Diener, H.C., Ellis, P., Hettiarachchi, J., Poole, P.H., Christianssen, I., Kleinermans, D., Kok, J.G. & Ferrari, M.D. (2000). No acute antimigraine efficacy of CP-122,288, a highly potent inhibitor of neurogenic inflammation: results of two randomized, double-blind, placebo-controlled clinical trials. *Ann Neurol*, **47,** 238-241.

Rorabaugh, B.R., Scofield, M.A., Smith, D.D., Jeffries, W.B. & Abel, P.W. (2001). Functional calcitonin gene-related peptide subtype 2 receptors in porcine coronary arteries are identified as calcitonin gene-related peptide subtype 1 receptors by radioligand binding and reverse transcription-polymerase chain reaction. *J Pharmacol Exp Ther*, **299,** 1086-1094.

Rosenfeld, M.G., Mermod, J.J., Amara, S.G., Swanson, L.W., Sawchenko, P.E., Rivier, J., Vale, W.W. & Evans, R.M. (1983). Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. *Nature*, **304,** 129-135.

Rossi, S.G., Dickerson, I.M. & Rotundo, R.L. (2003). Localization of the calcitonin gene-related peptide receptor complex at the vertebrate neuromuscular junction and its role in regulating acetylcholinesterase expression. *J Biol Chem*, **278,** 24994-25000.

Rothrock, J., North, J., Madden, K., Lyden, P., Fleck, P. & Dittrich, H. (1993). Migraine and migrainous stroke: risk factors and prognosis. *Neurology*, **43,** 2473-2476.

Roudenok, V. & Schmitt, O. (2001). Upregulation of vasoactive intestinal polypeptide (VIP) and calcitonin gene-related peptide (CGRP) expression in stellate ganglia of children with congenital cardiovascular lesions. *Ann Anat*, **183,** 209-212.

Rudner, X.L., Berkowitz, D.E., Booth, J.V., Funk, B.L., Cozart, K.L., D'Amico, E.B., El-Moalem, H., Page, S.O., Richardson, C.D., Winters, B., Marucci, L. & Schwinn, D.A. (1999). Subtype specific regulation of human vascular alpha(1)-adrenergic receptors by vessel bed and age. *Circulation*, **100,** 2336-2343.

Russell, M.B. & Olesen, J. (1996). A nosographic analysis of the migraine aura in a general population. *Brain*, **119 (Pt 2),** 355-361.

Saetrum Opgaard, O., de Vries, R., Tom, B., Edvinsson, L. & Saxena, P.R. (1999). Positive inotropy of calcitonin gene-related peptide and amylin on porcine isolated myocardium. *Eur. J. Pharmacol.*, **385,** 147-154.

Saetrum Opgaard, O., Hasbak, P., De Vries, R., Saxena, P.R. & Edvinsson, L. (2000). Positive inotropy mediated via CGRP receptors in isolated human myocardial trabeculae. *Eur. J. Pharmacol.*, **397,** 373-382.

Sams-Nielsen, A., Orskov, C. & Jansen-Olesen, I. (2001). Pharmacological evidence for CGRP uptake into perivascular capsaicin sensitive nerve terminals. *Br. J. Pharmacol.*, **132,** 1145-1153.

Sato, H., Kawashima, K., Yuki, M., Kazumori, H., Rumi, M.A., Ortega-Cava, C.F., Ishihara, S. & Kinoshita, Y. (2003). Lafutidine, a novel histamine H2-receptor antagonist, increases serum calcitonin gene-related peptide in rats after water immersion-restraint stress. *J Lab Clin Med*, **141,** 102-105.

Saxena, P.R. (1987). Arteriovenous anastomoses and veins in migraine research. In *Migraine, clinical, therapeutic, conceptual and research aspects*. ed BLAU, J.N. pp. 581-596. London, UK: Chapman and Hall medicin.

Saxena, P.R. (1990). Is there still a case for the shunt hypothesis in migraine? In *Migraine: a spectrum of ideas*. eds SANDLER, M. & COLLINS, G.M. pp. 191-199. Oxford: Oxford University Press.

Saxena, P.R. (1995). Cranial arteriovenous shunting, an *in vivo* animal model for migraine. In *Experimental headache models*. eds OLESEN, J. & MOSKOWITZ, M.A. pp. 189-198. Philadelphia, USA: Lippincott-Raven Publishers.

Saxena, P.R., De Vries, P., Heiligers, J.P., Bax, W.A., Maassen VanDenBrink, A. & Yocca, F.D. (1998). BMS-181885, a 5-HT$_{1B/1D}$ receptor ligand, in experimental models predictive of antimigraine activity and coronary side-effect potential. *Eur. J. Pharmacol.*, **351,** 329-339.

Saxena, P.R. & Den Boer, M.O. (1991). Pharmacology of antimigraine drugs. *J Neurol*, **238 Suppl 1,** S28-35.

Saxena, P.R. & Ferrari, M.D. (1989). 5-HT(1)-like receptor agonists and the pathophysiology of migraine. *Trends Pharmacol Sci*, **10,** 200-204.

Saxena, P.R., Schamhardt, H.C., Forsyth, R.P. & Hoeve, J. (1980). Computer programs for the radioactive microsphere technique. Determination of regional blood flows and other haemodynamic variables in different experimental circumstances. *Comput. Programs Biomed.*, **12,** 63-84.

Saxena, P.R. & Tfelt-Hansen, P. (1993). Sumatriptan. In *The headaches*. eds OLESEN, J., TFELT-HANSEN, P. & WELCH, K.M.A. pp. 329-341. New York: Raven Press Ltd.

Saxena, P.R. & Tfelt-Hansen, P. (2000). Triptans, 5-HT$_{1B/1D}$ receptor agonists in the acute treatment of migraine. In *The headaches*. eds OLESEN, J., TFELT-HANSEN, P. & WELCH, K.M.A. pp. 411-438. Philadelphia, USA: Lippincott Williams & Wilkins.

Saxena, P.R. & Verdouw, P.D. (1982). Redistribution by 5-hydroxytryptamine of carotid arterial blood at the expense of arteriovenous anastomotic blood flow. *J. Physiol. (Lond.)*, **332,** 501-520.

Schindler, M. & Doods, H.N. (2002). Binding properties of the novel, non-peptide CGRP receptor antagonist radioligand, [(3)H]BIBN4096BS. *Eur J Pharmacol*, **442,** 187-193.

Schuijt, M.P., de Vries, R., Saxena, P.R. & Danser, A.H. (1999). No vasoactive role of the angiotensin II type 2 receptor in normotensive Wistar rats. *J. Hypertens.*, **17,** 1879-1884.

Schwaag, S., Nabavi, D.G., Frese, A., Husstedt, I.W. & Evers, S. (2003). The association between migraine and juvenile stroke: a case-control study. *Headache*, **43,** 90-95.

Segond von Banchet, G., Pastor, A., Biskup, C., Schlegel, C., Benndorf, K. & Schaible, H.G. (2002). Localization of functional calcitonin gene-related peptide binding sites in a subpopulation of cultured dorsal root ganglion neurons. *Neuroscience*, **110,** 131-145.

Shen, Y.T., Pittman, T.J., Buie, P.S., Bolduc, D.L., Kane, S.A., Koblan, K.S., Gould, R.J. & Lynch, J.J., Jr. (2001). Functional role of alpha-calcitonin gene-related peptide in the regulation of the cardiovascular system. *J. Pharmacol. Exp. Ther.*, **298,** 551-558.

Shepheard, S.L., Williamson, D.J., Beer, M.S., Hill, R.G. & Hargreaves, R.J. (1997). Differential effects of 5-HT$_{1B/1D}$ receptor agonists on neurogenic dural plasma extravasation and vasodilation in anaesthetized rats. *Neuropharmacology*, **36,** 525-533.

Shepheard, S.L., Williamson, D.J., Williams, J., Hill, R.G. & Hargreaves, R.J. (1995). Comparison of the effects of sumatriptan and the NK1 antagonist CP-99,994 on plasma extravasation in Dura mater and c-fos *mRNA* expression in trigeminal nucleus caudalis of rats. *Neuropharmacology*, **34,** 255-261.

Sigrist, S., Franco-Cereceda, A., Muff, R., Henke, H., Lundberg, J.M. & Fischer, J.A. (1986). Specific receptor and cardiovascular effects of calcitonin gene-related peptide. *Endocrinology*, **119,** 381-389.

Silberstein, S.D. (1995). Migraine symptoms: results of a survey of self-reported migraineurs. *Headache*, **35,** 387-396.

Silberstein, S.D. & Lipton, R.B. (1994). Overview of diagnosis and treatment of migraine. *Neurology*, **44,** S6-16.

Silberstein, S.D. & Silberstein, M.M. (1990). New concepts in the pathogenesis of headache. Part II. In *Pain Manage*. pp. 334-342.

Smith, D., Hill, R.G., Edvinsson, L. & Longmore, J. (2002). An immunocytochemical investigation of human trigeminal nucleus caudalis: CGRP, substance P and 5-HT$_{1D}$-receptor immunoreactivities are expressed by trigeminal sensory fibres. *Cephalalgia*, **22,** 424-431.

Spierings, E.L. (2003). Pathogenesis of the migraine attack. *Clin J Pain*, **19,** 255-262.

Spierings, E.L. & Saxena, P.R. (1980). Effect of isometheptene on the distribution and shunting of 15 μM microspheres throughout the cephalic circulation of the cat. *Headache*, **20,** 103-106.

Spokes, R.A. & Middlefell, V.C. (1995). Simultaneous measurement of plasma protein extravasation and carotid vascular resistance in the rat. *Eur. J. Pharmacol.*, **281,** 75-79.

Steel, R.G.D. & Torrie, J.H. (1980). *A biomedical approach (2nd edition)*.

Steenbergh, P.H., Hoppener, J.W., Zandberg, J., Van de Ven, W.J., Jansz, H.S. & Lips, C.J. (1984). Calcitonin gene related peptide coding sequence is conserved in the human genome and is expressed in medullary thyroid carcinoma. *J Clin Endocrinol Metab*, **59,** 358-360.

Sternini, C., De Giorgio, R. & Furness, J.B. (1992). Calcitonin gene-related peptide neurons innervating the canine digestive system. *Regul. Pept.*, **42,** 15-26.

Stewart, W., Linet, M., Celentano, D., Van Natta, M. & Ziegler, D. (1993). Age and sex-specific incidence rates of migraine with and without visual aura. *Am J Epidemiol.*, **34,** 1111-1120.

Stewart, W.F., Lipton, R.B., Celentano, D.D. & Reed, M.L. (1992). Prevalence of migraine headache in the United States. Relation to age, income, race, and other sociodemographic factors. *Jama*, **267,** 64-69.

Stoll, A. (1920). Zur Kenntnis der Mutterkornalkaloide. *Verh. Naturf. Ges.*, **101,** 190-191.

Strecker, T., Dux, M. & Messlinger, K. (2002). Increase in meningeal blood flow by nitric oxide--interaction with calcitonin gene-related peptide receptor and prostaglandin synthesis inhibition. *Cephalalgia*, **22,** 233-241.

Strecker, T. & Messlinger, K. (2003). Neuropeptide release in the dura mater encephali in response to nitric oxide--relevance for the development of vascular headaches? *Schmerz*, **17,** 179-184.

Sun, R.Q., Lawand, N.B. & Willis, W.D. (2003). The role of calcitonin gene-related peptide (CGRP) in the generation and maintenance of mechanical allodynia and hyperalgesia in rats after intradermal injection of capsaicin. *Pain*, **104,** 201-208.

Sykes, R.M., Spyer, K.M. & Izzo, P.N. (1994). Central distribution of substance P, calcitonin gene-related peptide and 5-hydroxytryptamine in vagal sensory afferents in the rat dorsal medulla. *Neuroscience*, **59,** 195-210.

Szallasi, A. (2002). Vanilloid (capsaicin) receptors in health and disease. *Am J Clin Pathol*, **118,** 110-121.

Szallasi, A. & Blumberg, P.M. (1999). Vanilloid (capsaicin) receptors and mechanisms. *Pharmacol. Rev.*, **51,** 159-212.

Takami, K., Kawai, Y., Uchida, S., Tohyama, M., Shiotani, Y., Yoshida, H., Emson, P.C., Girgis, S., Hillyard, C.J. & MacIntyre, I. (1985). Effect of calcitonin gene-related peptide on contraction of striated muscle in the mouse. *Neurosci Lett*, **60,** 227-230.

Tepper, S.J., Rapoport, A.M. & Sheftell, F.D. (2002). Mechanisms of action of the 5-HT$_{1B/1D}$ receptor agonists. *Arch. Neurol.*, **59,** 1084-1088.

Tfelt-Hansen, P., De Vries, P. & Saxena, P.R. (2000). Triptans in migraine: a comparative review of pharmacology, pharmacokinetics and efficacy. *Drugs*, **60,** 1259-1287.

Thompson, W.H. (1894). Ergots. *J Nerv Ment Dis*, **21,** 124.

Tom, B., De Vries, P., Heiligers, J.P., Willems, E.W., Kapoor, K., John, G.W. & Saxena, P.R. (2002). Effects of donitriptan on carotid haemodynamics and cardiac output distribution in anaesthetized pigs. *Cephalalgia*, **22,** 37-47.

Ueda, T., Ugawa, S., Saishin, Y. & Shimada, S. (2001). Expression of receptor-activity modifying protein (RAMP) *mRNA*s in the mouse brain. *Brain Res Mol Brain Res*, **93,** 36-45.

Urban, L. & Dray, A. (1992). Synaptic activation of dorsal horn neurons by selective C-fibre excitation with capsaicin in the mouse spinal cord *in vitro*. *Neuroscience*, **47,** 693-702.

Ursell, P.C., Ren, C.L. & Danilo, P., Jr. (1991). Anatomic distribution of autonomic neural tissue in the developing dog heart: II. Nonadrenergic noncholinergic innervation by calcitonin gene-related peptide-immunoreactive tissue. *Anat Rec*, **230,** 531-538.

Van Gelderen, E.M., Du, X.Y., Schoemaker, R.G. & Saxena, P.R. (1995). Carotid blood flow distribution, haemodynamics and inotropic responses following calcitonin gene-related peptide in the pig. *Eur. J. Pharmacol.*, **284,** 51-60.

van Rossum, D., Hanisch, U.K. & Quirion, R. (1997). Neuroanatomical localization, pharmacological characterization and functions of CGRP, related peptides and their receptors. *Neurosci Biobehav Rev*, **21,** 649-678.

Velling, D.A., Dodick, D.W. & Muir, J.J. (2003). Sustained-release niacin for prevention of migraine headache. *Mayo Clin Proc*, **78,** 770-771.

Ventura, S., Lau, W.A., Buljubasich, S. & Pennefather, J.N. (2000). Calcitonin gene-related peptide (CGRP) inhibits contractions of the prostatic stroma of the rat but not the guinea-pig. *Regul. Pept.*, **91,** 63-73.

Verheggen, R., Bumann, K. & Kaumann, A.J. (2002). BIBN4096BS is a potent competitive antagonist of the relaxant effects of alpha-CGRP on human temporal artery: comparison with CGRP$_{(8-37)}$. *Br J Pharmacol*, **136,** 120-126.

Verheggen, R., Hundeshagen, A.G., Brown, A.M., Schindler, M. & Kaumann, A.J. (1998). 5-HT$_{1B}$ receptor-mediated contractions in human temporal artery: evidence from selective antagonists and 5-HT receptor *mRNA* expression. *Br J Pharmacol*, **124,** 1345-1354.

Villa, I., Dal Fiume, C., Maestroni, A., Rubinacci, A., Ravasi, F. & Guidobono, F. (2003). Human osteoblast-like cell proliferation induced by calcitonin-related peptides involves PKC activity. *Am J Physiol Endocrinol Metab*, **284,** E627-633.

Villalón, C.M., Centurión, D., Lujan-Estrada, M., Terron, J.A. & Sanchez-Lopez, A. (1997). Mediation of 5-HT-induced external carotid vasodilatation in GR 127935-pretreated vagosympathectomized dogs by the putative 5-HT7 receptor. *Br J Pharmacol*, **120,** 1319-1327.

Villalón, C.M., Centurión, D., Valdivia, L.F., De Vries, P. & Saxena, P.R. (2002). An introduction to migraine: from ancient treatment to functional pharmacology and antimigraine therapy. *Proc West Pharmacol Soc*, **45,** 199-210.

Villalón, C.M. & Terrón, J.A. (1994). Characterization of the mechanisms involved in the effects of catecholamines on the canine external carotid blood flow. *Can. J. Physiol. Pharmacol.*, **72,** 165.

Waugh, D.J., Bockman, C.S., Smith, D.D. & Abel, P.W. (1999). Limitations in using peptide drugs to characterize calcitonin gene-related peptide receptors. *J Pharmacol Exp Ther*, **289,** 1419-1426.

Welch, K.M. (1994). Relationship of stroke and migraine. *Neurology*, **44,** S33-36.

Welch, K.M. (2003). Concepts of migraine headache pathogenesis: insights into mechanisms of chronicity and new drug targets. *Neurol Sci*, **24 Suppl 2,** S149-153.

Wiesenfeld-Hallin, Z., Hokfelt, T., Lundberg, J.M., Forssmann, W.G., Reinecke, M., Tschopp, F.A. & Fischer, J.A. (1984). Immunoreactive calcitonin gene-related peptide and substance P coexist in sensory neurons to the spinal cord and interact in spinal behavioral responses of the rat. *Neurosci Lett*, **52,** 199-204.

Willems, E., De Vries, P., Heiligers, J.P. & Saxena, P.R. (1998). Porcine carotid vascular effects of eletriptan (UK-116,044): a new 5-HT$_{1B/1D}$ receptor agonist with anti-migraine activity. *Naunyn Schmiedebergs Arch Pharmacol*, **358,** 212-219.

Willems, E.W., Trion, M., De Vries, P., Heiligers, J.P.C., Villalón, C.M. & Saxena, P.R. (1999). Pharmacological evidence that α_1- and α_2-adrenoceptors mediate vasoconstriction of carotid arteriovenous anastomoses in anaesthetized pigs. *Br. J. Pharmacol.*, **127,** 1263-1271.

Willems, E.W., Valdivia, L.F., Saxena, P.R. & Villalon, C.M. (2001). The role of several alpha(1)- and alpha(2)-adrenoceptor subtypes mediating vasoconstriction in the canine external carotid circulation. *Br J Pharmacol*, **132,** 1292-1298.

Willems, E.W., Valdivia, L.F., Villalon, C.M. & Saxena, P.R. (2003). Possible role of alpha-adrenoceptor subtypes in acute migraine therapy. *Cephalalgia*, **23,** 245-257.

Williamson, D.J. & Hargreaves, R.J. (2001). Neurogenic inflammation in the context of migraine. *Microsc. Res. Tech.*, **53,** 167-178.

Williamson, D.J., Hargreaves, R.J., Hill, R.G. & Shepheard, S.L. (1997). Sumatriptan inhibits neurogenic vasodilation of dural blood vessels in the anaesthetized rat--intravital microscope studies. *Cephalalgia*, **17,** 525-531.

Wimalawansa, S.J. (1996). Calcitonin gene-related peptide and its receptors: molecular genetics, physiology, pathophysiology, and therapeutic potentials. *Endocr Rev*, **17,** 533-585.

Wimalawansa, S.J. (2001). Blood pressure and cardiovascular tone: role of CGRP family of peptides. *Scientific World Journal*, **1,** 32.

Wimalawansa, S.J. & MacIntyre, I. (1988). Calcitonin gene-related peptide and its specific binding sites in the cardiovascular system of rat. *Int J Cardiol*, **20,** 29-37.

Wisskirchen, F.M., Burt, R.P. & Marshall, I. (1998). Pharmacological characterization of CGRP receptors mediating relaxation of the rat pulmonary artery and inhibition of twitch responses of the rat vas deferens. *Br. J. Pharmacol.*, **123,** 1673-1683.

Wisskirchen, F.M., Gray, D.W. & Marshall, I. (1999). Receptors mediating CGRP-induced relaxation in the rat isolated thoracic aorta and porcine isolated coronary artery differentiated by h(alpha) CGRP(8-37). *Br J Pharmacol*, **128,** 283-292.

Wu, D., Doods, H., Arndt, K. & Schindler, M. (2002). Development and potential of non-peptide antagonists for calcitonin-gene-related peptide (CGRP) receptors: evidence for CGRP receptor heterogeneity. *Biochem Soc Trans*, **30,** 468-473.

Wu, D., Eberlein, W., Rudolf, K., Engel, W., Hallermayer, G. & Doods, H. (2000). Characterisation of calcitonin gene-related peptide receptors in rat atrium and vas deferens: evidence for a [Cys(Et)(2, 7)]hCGRP-preferring receptor. *Eur J Pharmacol*, **400,** 313-319.

Wu, D.-M., Van Zwieten, P.A. & Doods, H.N. (2001). Effects of calcitonin gene-related peptide and BIBN4096BS on myocardial ischaemia in anaesthetized rats. *Acta Pharmacol. Sin.*, **22,** 588-594.

Yallampalli, C., Chauhan, M., Thota, C.S., Kondapaka, S. & Wimalawansa, S.J. (2002). Calcitonin gene-related peptide in pregnancy and its emerging receptor heterogeneity. *Trends Endocrinol Metab*, **13,** 263-269.

Yu, X.J., Cutrer, F.M., Moskowitz, M.A. & Waeber, C. (1997). The 5-HT$_{1D}$ receptor antagonist GR-127,935 prevents inhibitory effects of sumatriptan but not CP-122,288 and 5-CT on neurogenic plasma extravasation within guinea pig dura mater. *Neuropharmacology*, **36,** 83-91.

Yu, X.J., Waeber, C., Castanon, N., Scearce, K., Hen, R., Macor, J.E., Chauveau, J., Moskowitz, M.A. & Chaveau, J. (1996). 5-Carboxamido-tryptamine, CP-122,288 and dihydroergotamine but not sumatriptan, CP-93,129, and serotonin-5-O-carboxymethyl-glycyl -tyrosinamide block dural plasma protein extravasation in knockout mice that lack 5-hydroxytryptamine1B receptors. *Mol Pharmacol*, **49,** 761-765.

Zaidi, M., Brain, S.D., Tippins, J.R., Di Marzo, V., Moonga, B.S., Chambers, T.J., Morris, H.R. & MacIntyre, I. (1990). Structure-activity relationship of human calcitonin-gene-related peptide. *Biochem J*, **269,** 775-780.

Ziegler, D.K. & Hassanein, R.S. (1990). Specific headache phenomena: their frequency and coincidence. *Headache*, **30,** 152-156.

Zimmermann, K., Reeh, P.W. & Averbeck, B. (2003). S(+)-flurbiprofen but not 5-HT(1) agonists suppress basal and stimulated CGRP and PGE(2) release from isolated rat dura mater. *Pain*, **103,** 313-320.