DEPLETION OF CALCITONIN GENE-RELATED PEPTIDE (CGRP) BY CAPSAICIN IN CEREBRAL ARTERIES

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Effects of capsaicin *in vivo* and *in vitro* on calcitonin gene-related peptide (CGRP)-containing nerves were examined in cerebral arteries. CGRP-like immunoreactive (CGRP-I) nerves were demonstrated in cerebral arteries of rats, guinea pigs and rabbits. The overall distribution of CGRP-I nerves was similar to that of substance P-containing nerves. Since the distribution of CGRP-I nerves of cerebral arteries among these species was the densest in guinea pigs, this animal was used to study the effect of several treatments on CGRP-I nerves. Catecholamine (CA)-fluorescence but not CGRP-I nerves was abolished by surgical sympathectomy or reserpine pretreatment. Pretreatment of the animal with capsaicin abolished CGRP-I nerves of cerebral arteries. CGRP-like immunoreactivity disappeared from cerebral arteries by *in vitro* incubation with capsaicin. Capsaicin treatment *in vivo* and *in vitro* did not affect CA-fluorescence nerves in these arteries. Since capsaicin selectively affects primary afferent nerves, it is suggested that CGRP is contained in sensory nerves. Capsaicin may be a valuable tool in elucidating the physiological or pathophysiological role of CGRP in cerebral circulation.

Keywords — capsaicin; calcitonin gene-related peptide; cerebral artery; catecholamine

Pharmacological studies *in vitro* have revealed the presence of vasodilator nerves which are distinct from classical adrenergic and cholinergic nerves in cerebral arteries.^{1–4)} Recently, several biologically active neuropeptides, such as vasoactive intestinal polypeptide (VIP), substance P and peptide with histidine and isoleucine (PHI), have been demonstrated in cerebrovascular nerves.^{5–8)} Although these peptides cause vasodilation when applied *in vitro*,^{8,9)} functional relations of the neuropeptides and neurogenic vasodilation remain to be determined.

Rat and human calcitonin genes have been shown to encode a novel peptide, calcitonin gene-related peptide (CGRP).^{10,11)} The presence of CGRP-like immunoreactivity and messenger ribonucleic acid (mRNA) for CGRP have been demonstrated in the central and peripheral nervous system.¹⁰⁾ Recently, CGRP was isolated from porcine spinal cord and was found to be a neuropeptide.¹²⁾ CGRP exerts several biological effects including vasodilation, cardioacceleration and inhibition of gastric acid secretion.^{13–15)} Besides such biological activities, it has been demonstrated that CGRP coexists with substance P in spinal and trigeminal ganglia.^{16–18)}

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), an active ingredient of hot peppers, is known to cause the depletion of substance P from a certain population of sensory nerves.¹⁹⁾ Capsaicin also depletes CGRP from nerves in the guinea pig heart.¹⁴⁾

Many neuropeptides have been demonstrated in the central and peripheral nervous system.^{20,21)} However, the lack of a specific antagonist to most of these neuropeptides hampered the determination of physiological roles of neuropeptides. Since a specific antagonist of CGRP is not available as yet, it is important to develop a pharmacological treatment which affects CGRP-containing nerves in elucidating the functional significance of the peptide. In the present study, the distribution of CGRP-containing nerves and the effect of capsaicin on these nerves were examined in cerebral arteries.

METHODS

Male Wistar rats (250–300 g), guinea pigs (300–350 g) and rabbits (3.0–3.5 kg) were anesthetized with Na-pentobarbital (50 mg/kg i.p.). The animals were exsanguinated and the brains were removed and transferred to ice-cold Krebs-Ringer solution. The composition of the

solution was as follows (mM): NaCl, 113; KCl, 4.8; CaCl₂, 2.2; KH₂PO₄, 1.2; MgCl₂, 1.2; NaHCO₃, 25 and glucose, 5.5. The circle of Willis, middle cerebral and basilar arteries were dissected out and remaining blood in the lumen was flushed.

Histochemistry — The blood vessels were fixed with paraformaldehyde and picric acid for 48 h at 4 °C and processed for fluorescence immunohistochemistry using whole mounts.²²⁾ In brief, the tissues were dehydrated in ethanol, cleared with xylene and rehydrated. The tissues were treated with either anti-CGRP antibodies raised in a rabbit (CRB, Cambridge England) or anti-substance P antibodies also raised in a rabbit (Immunonuclear Corp) at a dilution of 1:1600. The tissues were treated with biotinylated anti-rabbit immunoglobulin G (IgG) and then FITC-labelled avidin (Vector Labs.). The specimen was mounted in buffered glycerol and fluorescence was examined under a Zeiss fluorescence microscope. Antibodies preabsorbed with respective peptides (10 µM) served as controls. The antibodies used may crossreact with unknown peptides which share the immunoreactive amino acid sequences. Therefore the immunoreactive materials were referred to as CGRP-like or substance P-like in the present study.

Arteries were processed for catecholamine (CA)-fluorescence histochemistry in whole mounts.²²⁾ The fresh arteries were immersed in glyoxilic acid (20 mg/kg) at 4 °C for 30 min, dried on coverslips and heated in an oven for 6 min at 105 °C. The tissues were mounted in xylene and examined under a fluorescence microscope.

Animal Treatment — Capsaicin was dissolved in 10% tween 80-10% ethanol-80% saline (v/v/v) and injected to 4 guinea pigs at a dose of 25 mg/kg/d s.c. for 2 d. The animals were sacrificed 4 d after the second injection.^{14,23)} Reserpine was injected i.p. to 3 guinea pigs

twice; the first dose was 5 mg/kg and the second dose injected on the next day was 2.5 mg/kg. The animals were sacrificed 1 d after the second injection. Superior cervical ganglia of 3 guinea pigs were extirpated bilaterally under anesthesia with ketamine (35 mg/kg i.m.) and xylazine (5 mg/kg i.m.). The animals were sacrificed 1 week after the operation.

Capsaicin Treatment in Vitro — The isolated basilar arteries of guinea pigs were incubated in 50 ml of organ bath containing Krebs-Ringers solution maintained at 37 °C and gassed with 95% O₂ and 5% CO₂. The tissues were equilibrated for 15 min and capsaicin was applied to the bath. For *in vitro* experiments capsaicin was prepared in ethanol at a concentration of 0.1 M and further diluted with distilled water.

RESULTS

CGRP-like immunoreactive (CGRP-I) nerve fibers were detected in all regions of cerebral arteries of rats, rabbits and guinea pigs. However, the density of CGRP-I fibers differed in these species (Fig. 1). CGRP-I fibers consisted of bundles of fibers and of single varicose fibers. In cerebral arteries of guinea pigs, CGRP-I fibers were densely distributed in the circle of Willis, middle cerebral and basilar arteries. The fibers distributed as meshwork in these arteries. In the circle of Willis and middle cerebral arteries of rats and rabbits, bundles and fine varicose fibers of CGRP-I nerves were distributed. The distribution of CGRP-I fibers in basilar arteries of rats and rabbits were relatively sparse (Table I). There were numerous substance P-like immunoreactive (substance P-I) nerves as reported by Edvinsson *et al.*⁶⁾ The overall distribution of CGRP-I nerves were similar to that of substance P-I nerves (data not shown).

In guinea pigs pretreated with capsaicin (total 50 mg/kg), CGRP-I nerves were not detected in the cerebral arteries (Fig. 2). Sub-

TABLE I. Frequency of CGRP-I Nerves in Cerebral Arteries of Rats, Rabbits and Guinea Pigs^{a)}

	Circle of Willis	Middle cerebral artery	Basilar artery
Rats	+	+	+
Rabbits	+	+	±
Guinea pigs	++	++	++

a) Frequency of nerve fibers was graded arbitrarily. ++: numerous fibers, +: moderate number of fibers, ±: few fibers.

FIG. 1. CGPR-I Nerves in Cerebral Arteries

A: Circle of Willis of the rat. B: Circle of Willis of the rabbit. C: Middle cerebral artery of the guinea pig. Bar equals 100 μ m.

stance P-I nerves were also not detected in cerebral arteries of capsaicin-pretreated guinea pigs (data not shown). On the contrary, there was dense mesh-like distribution of CA-fluorescence nerves in cerebral arteries of both control and capsaicin-treated guinea pigs. Similar distribution of CGRP-I nerves but not CA-fluorescence nerves were detected in cerebral arteries of guinea pigs pretreated with reserpine or undergone surgical sympathectomy.

Following the incubation of cerebral arteries *in vitro* with capsaicin (1 μ M) for 15 min at 37 $^{\circ}$ C, the intensity of CGRP-like immunoreactivity was markedly reduced (Fig. 3). Since most of fibers exhibited faint fluorescence, the distribution of fibers appeared sparser in capsaicin-treated tissue than in the control tissue. There were no differences in the intensity and distribution of CA-fluorescence nerves of cerebral arteries between the tissues incubated with and with-

FIG. 2. Effect of *in Vivo* Pretreatment of Guinea Pigs with Capsaicin on CGRP-I (A and B) and CA-Fluorescence (C and D) Nerves in the Circle of Willis

A and C are from control animals, B and D are from capsaicin-pretreated animals. Bar equals 100 μ m.

out capsaicin *in vitro*.

DISCUSSION

CGRP has been demonstrated to exert a potent vasodilatory action in rat aorta.¹³⁾ Demonstration of relatively dense distribution of CGRP-I nerves in cerebral arteries suggests that CGRP may have a physiological role in cerebral circulation. Neurogenic vasodilation induced by transmural nerve stimulation (TNS) *in*

vitro is mediated by nonadrenergic, noncholinergic nerves in cerebral arteries.^{3,4)} The neurotransmitter mechanism of the neurogenic vasodilation is not fully understood as yet. Therefore, the neurotransmitter role of CGRP in TNS-induced vasodilation remains to be determined.

Ultrastructural studies have shown that there are at least two types of nerve terminals around cerebral arteries; granular vesicle-containing nerves (GVN) and agranular vesicle-containing

FIG. 3. *Effect of in Vitro Treatment with Capsaicin on the Distribution of CGRP-I (A and B) and CA-Fluorescence (C and D) Nerves in the Guinea Pig Basilar Arteries*
A and C are from control tissues, B and D are from tissues incubated with capsaicin ($1 \mu\text{M}$ for 15 min) in vitro. Bar equals $100 \mu\text{m}$.

nerves (AVN).²⁴⁻²⁸) Following superior cervical ganglionectomy, GVN disappear while the distribution of AVN remains unchanged in cerebral arteries of cats and rabbits.^{25,29}) Therefore, AVN are of nonsympathetic nerve terminals. In the present study, CGRP-I nerves were detected in surgically sympathectomized or reserpine-pretreated tissues. Total disappearance of CA-fluorescence nerves following superior cervical

ganglionectomy indicates that norepinephrine-containing nerves of large cerebral arteries originate from superior cervical ganglia. The regional and species variation of the distribution of CGRP-I nerves was similar to that of AVN.^{25,26,28}) Therefore it is indicated that CGRP-I nerves are distinct from sympathetic nerves.

The distribution of CGRP-I nerves was simi-

lar to that of substance P-I nerves. This coincides with the demonstration of co-existence of CGRP and substance P in trigeminal and spinal ganglia.^{16-18,30} Capsaicin, a sensory neurotoxin, has been demonstrated to deplete substance P in cerebral arteries.³¹ In the present study, since capsaicin depleted both substance P and CGRP but not norepinephrine, it is suggested that CGRP and substance P co-exist in the same nerves in cerebral arteries. Capsaicin causes degeneration of a certain population of primary afferent neurons.¹⁹ This suggests that CGRP is contained in sensory nerves.

The mechanism of depletion of neuropeptides by capsaicin has not yet been determined. Capsaicin depleted CGRP not only by *in vivo* injection but also by *in vitro* application. No neuronal cell bodies have been demonstrated in the adventitial layer of cerebral arteries.^{24,25} Therefore, it is likely that capsaicin acts on nerve axons or terminals rather than on cell bodies and depletes neuropeptides. It has been demonstrated that capsaicin causes the release of substance P and somatostatin from the central and peripheral terminals of the primary afferent neurons.³²⁻³⁴ Furthermore capsaicin inhibits axonal transport of peptides.³⁵ Therefore the depletion of CGRP and substance P by capsaicin might be the result of their release from the nerves.

Although the physiological role is not yet elucidated, the existence of CGRP-containing nerves has been demonstrated in the central and peripheral nervous system.¹⁰ The neuronal release of CGRP by depolarizing stimuli through Ca^{2+} -dependent mechanism has been demonstrated.³⁶ CGRP exerts a wide variety of biological effects.^{13,14,37} Since a specific antagonist of CGRP is not available yet, capsaicin may be a valuable tool for elucidating the physiological role of CGRP in cerebral circulation.

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