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Local neurogenic regulation of rat hindlimb circulation: role of calcitonin gene-related peptide in vasodilatation after skeletal muscle contraction

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1 The mechanism of neurogenic regulation of skeletal muscle circulation was studied in the hindlimb of anaesthetized rats *in vivo*. Regional blood flow (RBF) of the hindlimb was recorded with a pulsed Doppler flow probe positioned in the iliac artery.

2 A short period (1 min) of sciatic nerve stimulation at 10 Hz caused a sustained increase in RBF (from 2.0 ± 0.2 to 3.7 ± 0.2 kHz at the peak), but no appreciable change in either MBP or HR, suggesting that the nerve stimulation produced local vasodilatation of the peripheral vasculature. The hyperaemic response reached a peak within 15 s and characteristically remained above the basal level for more than 5 min after the cessation of nerve stimulation. The response was regarded as a secondary response brought about by the contraction of skeletal muscles since (+)-tubocurarine (0.73 μmol kg^{-1}, i.a.) almost abolished it.

3 Lignocaine (43 μmol kg^{-1}, i.a.) and capsaicin (0.33 μmol kg^{-1}, i.a.) significantly suppressed the hyperaemic response to skeletal muscle contraction, suggesting that capsaicin-sensitive sensory nerves contribute to the hyperaemia. In contrast, an inhibitor of NO synthase, N_ω-nitro-L-arginine methyl ester (1 μmol kg^{-1} min^{-1}, i.v.), did not affect the hyperaemic response.

4 Serum levels of calcitonin gene-related peptide (CGRP) in iliac venous effluent significantly increased from 51 ± 4 to 77 ± 5 fmol ml^{-1} during the hyperaemic response to skeletal muscle contraction. A bolus injection of CGRP (300 pmol kg^{-1}, i.a.) induced a long-lasting increase in RBF of the hindlimb. Moreover, CGRP(8–37) (100 nmol kg^{-1} min^{-1}, i.v.), a specific $CGRP_1$ receptor antagonist, significantly suppressed the hyperaemic response, especially the sustained phase of the response which was almost abolished by this antagonist.

5 These results suggest that CGRP, which is released from peripheral endings of capsaicin-sensitive sensory nerves, partly mediates the hyperaemia evoked by skeletal muscle contraction of the rat hindlimb.

Keywords: Calcitonin gene-related peptide; capsaicin; sensory nerve; skeletal muscle; hyperaemia

Introduction

The hyperaemia that occurs when quiescent skeletal muscles are stimulated to contract represents one of the largest increases in perfusion seen in the tissues of normal mammals (Gaskell, 1877). A number of studies have proposed a contribution to this response by metabolic mediators such as oxygen, hydrogen, potassium, osmolality, adenine nucleotides, adenosine and inorganic phosphate (Haddy & Scott, 1975). In addition to metabolites, a contribution of peripheral nerves to the hyperaemic response after skeletal muscle contraction has also been postulated by Hilton (1953) and Honig & Frierson (1976). These studies provide evidence that the hyperaemic response is reduced or abolished by local anaesthetic agents such as procaine or lignocaine in cat gastrocnemius muscles (Hilton, 1953) and dog gracilis muscles (Honig & Frierson, 1976). However, the precise mechanism for the hyperaemic response mediated by nerve excitation remains to be elucidated.

Recent immunohistochemical studies have shown extensive perivascular localization of certain neuropeptides, calcitonin gene-related peptide (CGRP), substance P (SP) and vasoactive intestinal peptide (VIP), which are mostly contained in nerve terminals (Edvinsson *et al*, 1989). These peptides are known to be potent vasodilators (Bell & McDermott, 1996). It has also been demonstrated that peripheral nerve stimulation of sensory C-fibres leads to the release of CGRP and SP, thereby resulting in local vasodilatation and increased blood flow (Kawasaki *et al*., 1988; Gustafsson *et al*., 1994). Treatment with capsaicin, which depletes the CGRP and SP content of sensory nerves (Holzer, 1991; Maggi, 1991), can abolish the vasodilatation (Kawasaki *et al*., 1988). Recently, Kurozawa *et al*. (1991) showed that treatment with capsaicin largely suppressed the increase in forearm skin blood flow during graded exercise in man. Although they did not investigate further characteristics of the response, this finding led to the hypothesis that vasodilator neurotransmitters released from capsaicin-sensitive sensory nerves may be involved in the mechanism of hyperaemia after skeletal muscle contraction.

The objective of this study was to elucidate the neuronal mechanisms of the hyperaemic response associated with skeletal muscle contraction in the rat hindlimb. The experiments sought to clarify the contribution of capsaicin-sensitive sensory nerves to the hyperaemia and to identify plausible candidates for the neurotransmitter substance.

Methods

General procedure

Male Wistar rats (260–350 g; Charles River, Kanagawa, Japan) were used for *in vivo* experiments. Each animal was anaesthetized with urethane (17 mmol kg^{-1}, i.p.), placed on a water-perfused heating pad set at 37°C to maintain a constant body temperature, and artificially ventilated at a stroke volume of 3 ml and a rate of 50 cycles min^{-1} with room air by a respirator (Harvard Rodent Ventilator Model 683, U.S.A.) through an endotracheal tube inserted in the airway. Systemic

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blood pressure (BP), mean blood pressure (MBP) and heart rate (HR) were monitored continuously via a heparin-treated (100 u ml^{-1}) saline-filled catheter inserted in the right carotid artery and connected to a pressure transducer (Nihon Kohden DX-312, Tokyo, Japan). The right femoral vein was cannulated for administration of drugs and saline. A catheter, the tip of which was located about 2 mm proximal to the aortic origin of the iliac artery, was retrogradely inserted into the right iliac artery for intra-arterial injection of drugs into the left iliac artery.

A Doppler flow probe (0.5 mm in diameter) was positioned on the trunk of the left iliac artery and appropriately fixed so that the basal level of regional blood flow (RBF) did not drift during the contraction of hindlimb muscles. The probe was connected to a pulsed Doppler velocimeter (Crystal Biotech PD-20, Holliston, MA, U.S.A.) to allow continuous recording of RBF of the left hindlimb as a shift in kHz of Doppler signals. We compared values of RBF/MBP at the baseline and the peak of hyperaemia, which reflect changes in tension of the resistance vessels.

Under urethane anaesthesia, the recorded parameters, especially MBP, were unsteady. We found that treatment with propranolol, a β-adrenoceptor antagonist, improved this; the treatment changed MBP, HR and RBF from 105 ± 2 mmHg, 364 ± 5 beats min^{-1} and 5.6 ± 0.4 kHz to 130 ± 3 mmHg, 268 ± 3 beats min^{-1} and 3.0 ± 0.3 kHz ($n = 23$), respectively, and thereafter the parameters were maintained in a stable state for several hours. It is thus likely that under urethane anaesthesia, the sympathetic tone was considerably elevated, which made the parameters unstable. All experiments were therefore performed after treatment with propranolol (4 μmol kg^{-1}, i.v.). Each animal was left for more than 20 min before the experiments were commenced.

The left sciatic nerve was exposed at the lumbar level, ligated and sectioned. A pair of platinum electrodes was placed on the peripheral section of the sciatic nerve. The stimulating electrode and the sciatic nerve were insulated with warmed liquid paraffin. Electrical nerve stimulation was conducted with repeated square-wave pulses of 1 ms duration generated by a stimulator (Nihon Kohden SEN-7103) with an isolator (Nihon Kohden SS-201J) for 1 min. The threshold intensity required to produce hindlimb contractions, which was determined by macroscopic observation, was around 3 V.

Measurement of P_{CO_2}

Partial pressure of CO_2 (P_{CO_2}) of the venous blood was measured for the quantitative evaluation of skeletal muscle work elicited by sciatic nerve stimulation. About 0.2 ml of venous blood was collected through a catheter inserted in the left iliac vein, before and 1 min after sciatic nerve stimulation. Immediately after the blood had been collected, P_{CO_2} of the venous blood was measured with an automatic blood gas analyser (Ciba Corning 238, Tokyo, Japan).

Radioimmunoassay

Serum levels of neuropeptides (CGRP, VIP and SP) were measured by radioimmunoassay (RIA). About 0.35 ml of venous blood was collected through the catheter inserted in the left iliac vein 1 min after sciatic nerve stimulation. In some experiments, the same volume of arterial blood was collected through the catheter inserted in the right iliac artery. The collected blood was mixed with aprotinin (300 iu ml^{-1}) and EDTA-2Na (2 mg ml^{-1}) and centrifuged immediately (2000 $\times g$, 15 min), and the serum obtained was stored at $-80°C$ until assayed. Only one blood sample was taken from each rat. Serum neuropeptide-like immunoreactivity (LI) level was determined by RIA according to the method described previously (Fujimori *et al*., 1989). Rabbit antisera against rat α-CGRP, SP and VIP were purchased from Peninsula (Belmont, CA, U.S.A.).

Statistics

Data are presented as mean $\pm$ s.e.mean. Comparisons were made by one-way analysis of variance (ANOVA) followed by the Bonferroni method or Student's t test for unpaired data. A probability of $P < 0.05$ was accepted as the level of statistical significance.

Drugs

Adenosine, tetrodotoxin and (+)-tubocurarine chloride were purchased from Wako Chemicals (Osaka, Japan); atropine sulphate from Tanabe (Osaka, Japan); rat α-calcitonin gene-related peptide (CGRP), human α-CGRP(8–37), substance P (SP) and human vasoactive intestinal peptide (VIP) from Peptide Institute (Osaka, Japan); lidocaine from Teikoku Chemicals (Osaka, Japan); and adenosine, capsaicin, N_ω-nitro-L-arginine methyl ester (L-NAME) and propranolol from Sigma (St, Louis, MO, U.S.A.).

Capsaicin was dissolved in a solution containing 10% ethanol, 10% Tween 80, and 80% saline. CGRP(8–37) was dissolved in 0.05% bovine serum albumin with phosphate-buffered saline. Other drugs were dissolved in saline. L-NAME and CGRP(8–37) were continuously infused at a rate of 0.3 ml min^{-1} with a syringe pump (Nihon Kohden CFV-2100). CGRP and SP were administered as bolus injections of about 30 µl. Other drugs were administered as bolus injections of about 300 μl. Saline (0.1 ml) was used to flush the catheter. Appropriate vehicle controls showed no effect.

Results

Skeletal muscle contraction and hyperaemia

To produce hindlimb muscle contraction, the sciatic nerve was stimulated at a voltage of 6 V with square-wave pulses of 1 ms duration at several frequencies for 1 min. At a frequency of 1 or 2.5 Hz, sciatic nerve stimulation induced twitch contractions of the hindlimb muscles and only a slight increase in RBF (data not shown). Electrical stimulation at a frequency of 5 or 10 Hz elicited both hindlimb muscle contractions and a marked increase in RBF. The hyperaemic response reached a peak within 15 s and characteristically remained above the basal level for more than 5 min after the cessation of nerve stimulation (Figure 1). Sciatic nerve stimulation elicited an increase in RBF (from 2.0 ± 0.2 to 3.7 ± 0.2 kHz at the peak), whereas no appreciable change in either MBP or HR (105 ± 5 mmHg, 281 ± 10 beats min^{-1} before and 104 ± 6 mmHg, 281 ± 10 beats min^{-1} after nerve stimulation) was observed ($n = 23$, Figure 1). The nerve stimulation also significantly increased P_{CO_2} of the venous blood in the hindlimb (Table 1).

Then (+)-tubocurarine (0.73 μmol kg^{-1}), a selective antagonist for muscle nicotinic acetylcholine receptors (Jenkinson, 1960), was injected intravenously 10 min before sciatic nerve stimulation, nerve stimulation at 10 Hz for 1 min caused neither contractions of the hindlimb nor an increase in P_{CO_2} of venous blood (Figure 1, Table 1). Simultaneously, the hyperaemic response to sciatic nerve stimulation was abolished (Figures 1 and 2). Since (+)-tubocurarine is a specific blocker at the neuromuscular junction (Jenkinson, 1960), the hyperaemia was regarded as a secondary response brought about by skeletal muscle contraction, i.e., active hyperaemia.

Several previous studies, which suggested the contribution of peripheral nerves to active hyperaemia, showed that the hyperaemia is reduced or abolished by a local anaesthetic agent such as procaine or lignocaine (Hilton, 1953; Honig & Frierson, 1976). We therefore assessed the effects of lignocaine on active hyperaemia in rat hindlimb. Lignocaine (43 μmol kg^{-1}, i.a.) significantly attenuated the hyperaemic response to 1 min, 10 Hz sciatic nerve stimulation (Figure 2). That is, at least half of the hyperaemic response produced by

1 min, 10 Hz sciatic nerve stimulation appeared to result from a lignocaine-sensitive mechanism. The sciatic nerve stimulation still caused hindlimb contraction and increased P_{CO_2} of the venous effluent (Table 1), suggesting that lignocaine suppressed the hyperaemia without affecting motor neurones or skeletal muscle. The action of lignocaine on active hyperaemia seemed to be relatively specific, since reactive hyperaemia following occlusion of the abdominal aorta for 1 min was not affected by the same dose of lignocaine; % increase in RBF/MBP with reactive hyperaemia was $215 \pm 13\%$ of the basal value before and $207 \pm 16\%$ after treatment with lignocaine ($n = 6$). In the following experiments, we used this condition for sciatic nerve stimulation, i.e., at a frequency of 10 Hz and for a duration of 1 min, to explore the mechanism of neurogenic active hyperaemia.

Contribution of sensory nerves to active hyperaemia

Capsaicin at high doses is known to produce degenerative changes in C- and Aδ-primary sensory nerves (Holzer, 1991; Maggi, 1991). Approximately 20 min after control recording of the hyperaemic response, capsaicin (0.33 μmol kg^{-1}, i.a.) was injected as a bolus. Capsaicin induced a marked increase in RBF, which reached the maximum level within 40–50 min, then gradually decreased, and reached a plateau level, which was still significantly higher than the basal level, in 70–90 min. HR and MBP transiently increased and decreased, respectively, after the injection of capsaicin and returned to basal levels within 5 min. Sciatic nerve stimulation did not cause a significant increase in RBF/MBP 90 min after the injection of capsaicin (Figure 2b). In contrast, neither the increase in RBF/MBP elicited by aortic occlusion for 1 min, i.e., reactive hyperaemia, nor that induced by exogenously applied CGRP (300 pmol kg^{-1}) was affected by capsaicin (Figure 2b). These results suggest that the inhibitory effects of capsaicin on active hyperaemia were specific and unrelated to the increased basal level of RBF by capsaicin. An alternative possibility is that capsaicin inhibited skeletal muscle contractions, which in turn reduced the hyperaemic response. However, this possibility was ruled out by the finding that the sciatic nerve stimulation-induced increase in P_{CO_2} of the venous effluent, which had a good correlation with the level of muscle work, was not affected by treatment with capsaicin. These results support the hypothesis that capsaicin-sensitive sensory nerves contribute to active hyperaemia.

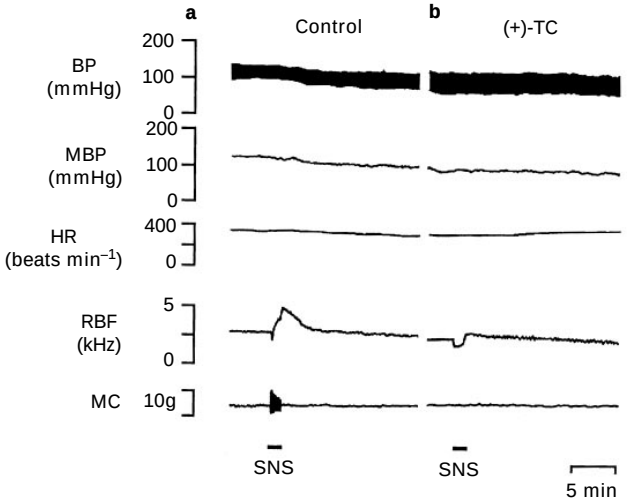

Figure 1 Typical traces of systemic blood pressure (BP), mean blood pressure (MBP), heart rate (HR) and regional blood flow (RBF) of the iliac artery recorded before (a) and after (b) treatment with (+)-tubocurarine ((+)-TC; 0.73 μmol kg^{-1}, i.v.). Electrical stimulation of the sciatic nerve (SNS) at 10 Hz for 1 min caused hindlimb muscle contractions (MC) and an increase in RBF without any changes in MBP and HR. Approximately 20 min after the control recording, (+)-tubocurarine was injected as a bolus into the femoral vein. After 10 min, SNS was imposed again. Hindlimb muscle contractions were recorded with a force transducer via a thread tied to the ankle of the left hindlimb.

Identification of the mediator of neurogenic active hyperaemia

Previous studies (Kawasaki *et al*., 1988; Gustafsson *et al*., 1994) have suggested that peripheral nerve stimulation of sensory C-fibres leads to the release of CGRP and SP, which are known to be potent vasodilators (Burnstock, 1990; Lundberg, 1996). We therefore measured changes in levels of these neuropeptides in the venous effluent by RIA. Sciatic nerve stimulation significantly increased serum CGRP-LI level in iliac venous blood (Figure 3a), while the level in arterial blood was not changed (48.0 ± 6.2 fmol ml^{-1} before and 58.8 ± 5.9 fmol ml^{-1} after sciatic nerve stimulation; $n = 10$). Serum CGRP-LI level in iliac venous blood increased after bolus administration of capsaicin (0.33 μmol kg^{-1}, i.a.), and the level after 90 min was still above the basal level (Figure 3b). Sciatic nerve stimulation under these conditions failed to cause any increase in the serum CGRP-LI level (Figure 3b). Similarly, when (+)-tubocurarine (0.73 μmol kg^{-1}, i.v.), which abolished active hyperaemia, was pre-injected as a bolus, sciatic nerve stimulation did not cause any change in serum CGRP-LI level in venous blood (Figure 3b), suggesting that skeletal muscle contraction initiates the release of CGRP. A small, but significant, increase in serum SP-LI level in venous blood was also observed after sciatic nerve stimulation (Figure 3a). In contrast, sciatic nerve stimulation did not cause any change in serum VIP-LI level (Figure 3a).

We next examined the effects of neuropeptides on RBF and compared the responses with active hyperaemia. As shown in Figure 4, a bolus intra-arterial injection of CGRP (300 pmol kg^{-1}, i.a.) induced a long-lasting (more than 5 min)

Table 1 Sciatic nerve stimulation (SNS)-induced changes in partial pressure of CO_2 (P_{CO_2}) before and after treatment with various drugs

Drugs		P_{CO_2} (mmHg) *Before SNS*	*After SNS*	n
(+) -Tubocurarine	Before	27.2 ± 2.8	59.5 ± 4.8*	6
	After	32.2 ± 2.1	36.3 ± 4.5†	
Lignocaine	Before	31.7 ± 3.7	72.6 ± 10.4*	5
	After	34.1 ± 2.2	66.7 ± 7.5*	
Capsaicin	Before	37.8 ± 3.6	59.3 ± 3.8*	5
	After	37.2 ± 2.8	64.0 ± 3.8*	
CGRP(8–37)	Before	30.5 ± 2.3	53.1 ± 3.5*	5
	After	34.6 ± 7.0	66.2 ± 5.5*	

Values are given as mean $\pm$ s.e.mean. *Significant difference at $P < 0.05$ compared with value before SNS. †Significant difference at $P < 0.05$ compared with value in the absence of drug. Comparisons were made by use of ANOVA followed by the Bonferroni method. *n*: Number of rats.

increase in RBF of the hindlimb with only small changes in HR or BP, the time course of which was quite similar to that of active hyperaemia. A similar response was produced by VIP (300 pmol kg^{-1}, i.a.). In contrast, the hyperaemic response to exogenously applied SP (200 pmol kg^{-1}, i.a.) was transient and lasted for only about 30 s (Figure 4). Similarly, adenosine (100 nmol kg^{-1}, i.a.), which is known to be one of the endogenous vasodilators participating in active hyperaemia in various tissues (Haddy, 1975), caused a transient increase in RBF (Figure 4). We also found that the hyperaemic response to CGRP (300 pmol kg^{-1}, i.a.) was not affected by continuous infusion of L-NAME (1 μmol kg^{-1} min^{-1}, i.v.; Figure 5). Similarly, the hyperaemia elicited by sciatic nerve stimulation was not affected by treatment with L-NAME (Figure 5). In contrast, the increase in vascular conductance by SP (200 pmol kg^{-1}, i.a.), which has been shown to cause NO-dependent vasorelaxation (Persson *et al*., 1991), was significantly suppressed by the same dose of L-NAME (Figure 5).

To explore the possible involvement of CGRP in the response to sciatic nerve stimulation, the effect of CGRP receptor antagonist, CGRP(8–37), was examined. We found that continuous infusion of CGRP(8–37) (100 nmol kg^{-1} min^{-1}, i.v.) significantly reduced the hyperaemic response to sciatic nerve stimulation (Figure 6a). It was of interest that CGRP(8–37) preferentially attenuated the sustained response (Figure 6b). Figure 6c illustrates the time course of the CGRP(8–37)-sensitive hyperaemic response, which was determined by subtracting the response in the presence of

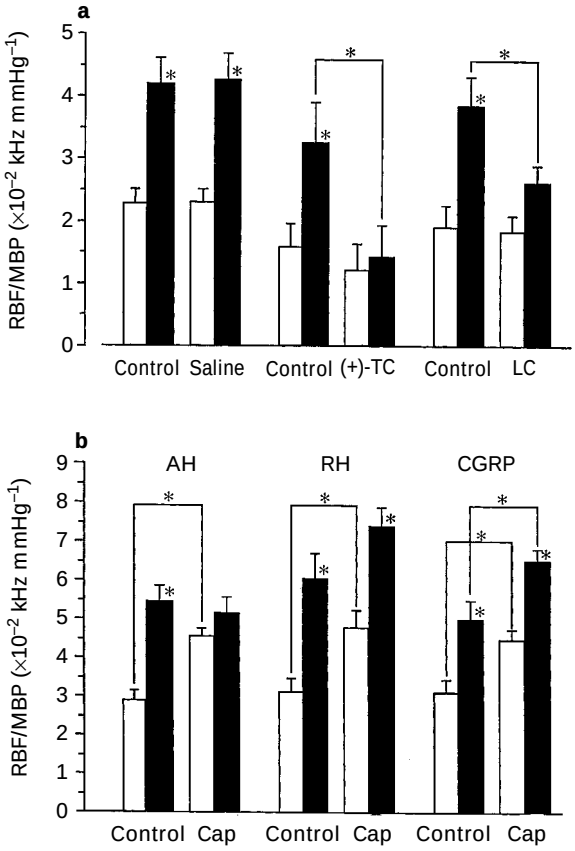

Figure 2 Effects of various drugs on active hyperaemia induced by sciatic nerve stimulation. (a) Sciatic nerves were stimulated before (Control) and approximately 10 min after a bolus injection of saline (1 mg kg^{-1}, i.v.), (+)-tubocurarine ((+)-TC; 0.73 μmol kg^{-1}, i.v.) or lignocaine (LC; 43 μmol kg^{-1}, i.a.). (b) Active hyperaemia (AH), reactive hyperaemia (RH) or hyperaemic response to CGRP (300 pmol kg^{-1}, i.a.) was induced before (Control) and 90 min after a bolus injection of capsaicin (Cap; 0.33 μmol kg^{-1}, i.a.). Open and solid columns represent values at the baseline and at the peak of the hyperaemic response, respectively. Data are expressed as mean ± s.e.mean of values obtained from six to nine rats. $*P<0.05$ compared with baseline or respective control (ANOVA followed by the Bonferroni method).

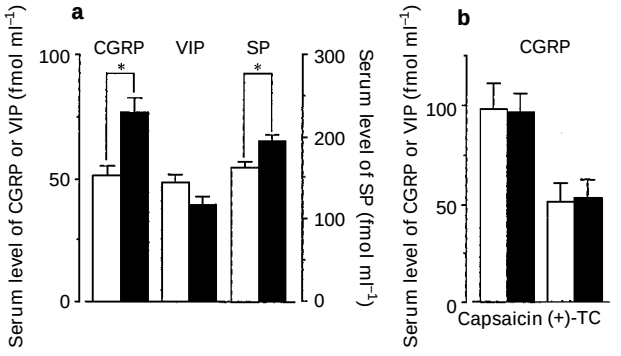

Figure 3 Changes in serum levels of neuropeptides in the venous effluent. (a) Serum levels of calcitonin gene-related peptide (CGRP)-, vasoactive intestinal peptide (VIP)-, and substance P (SP)-like immunoreactivity (LI) before (open columns) and after (solid columns) sciatic nerve stimulation at 10 Hz for 3 min. (b) Serum GCRP-LI level before (open columns) and after (solid columns) sciatic nerve stimulation in rats pretreated with capsaicin (0.33 μmol kg^{-1}, i.a.) or (+)-tubocurarine ((+)-TC; 0.73 μmol kg^{-1}, i.v.). Data are expressed as mean ± s.e.mean of values obtained from six to nine rats. $*P<0.05$ compared with respective control (Student's *t* test).

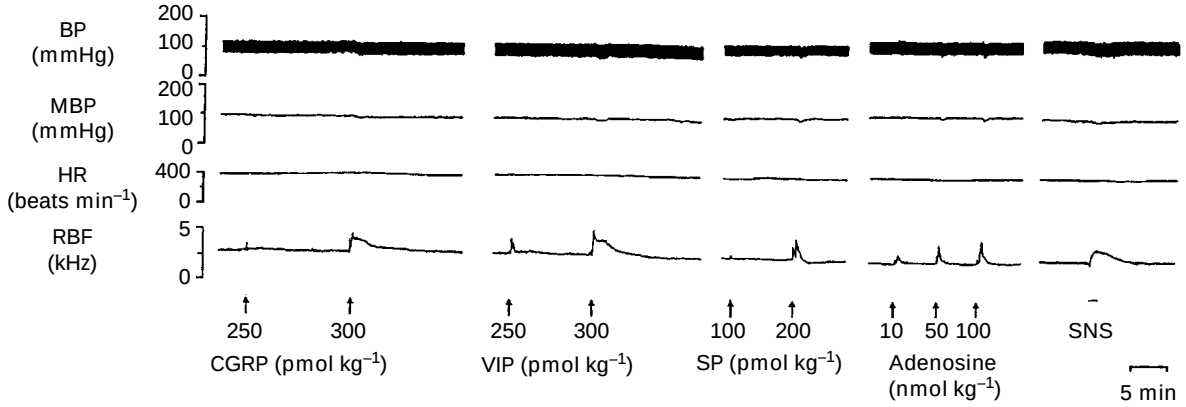

Figure 4 Typical traces of the effects of intra-arterial injection of calcitonin gene-related peptide (CGRP; 250, 300 pmol kg^{-1}), vasoactive intestinal peptide (VIP; 250, 300 pmol kg^{-1}), substance P (SP; 100, 200 pmol kg^{-1}) and adenosine (10, 50, 100 nmol kg^{-1}) and sciatic nerve stimulation (SNS; 10 Hz, 1 min) on systemic blood pressure (BP), mean blood pressure (MBP), heart rate (HR) and regional blood flow (RBF) of the iliac artery.

CGRP(8–37) from the control, showing a sustained response to endogenously released CGRP during active hyperaemia. The same dose of CGRP(8–37) almost abolished the hyperaemic response to CGRP (300 pmol kg^{-1}, i.a.) ($n=6$, data not shown). In contrast, CGRP(8–37) (100 nmol kg^{-1} min^{-1}, i.v.) did not affect the hyperaemic response to SP (200 pmol kg^{-1}, i.a.) or VIP (300 pmol kg^{-1}, i.a.) ($n=3$, data not shown). These observations suggest a direct link between CGRP and active hyperaemia, especially the sustained response.

Discussion

The present study showed that the concentration of rat hindlimb muscles elicited by sciatic nerve stimulation produced a long-lasting increase in RBF measured with a pulsed Doppler flow probe, which was due to local vasodilatation of the peripheral vasculature. More than forty years ago, Hilton (1953) found that active hyperaemia was sensitive to local anaesthetic agents and postulated a contribution to active hyperaemia of peripheral nerves other than sympathetic nerves. Although this finding has been supported by several investigators (Honig & Frierson, 1976), the detailed mechanism remains unclear. The present study confirmed these findings in rat hindlimb and provide further evidence that sensory nerves and their neurotransmitter CGRP are involved in the mechanisms producing active hyperaemia after a short period of hindlimb muscle contraction.

In the present study, we found that the hyperaemia elicited by sciatic nerve stimulation was partly suppressed by capsaicin. Capsaicin has selective neurotoxic effects on unmyelinated C- and small myelinated Aδ-primary sensory nerves (Holzer, 1991). Thus, it is likely that capsaicin-sensitive sensory nerves contribute to active hyperaemia. Recently, Edvinsson *et al.* (1989) revealed that VIP-LI, SP-LI and CGRP-LI nerve fibres are distributed widely around blood vessels of the rat hindlimb (Edvinsson *et al.*, 1989). SP and CGRP have been shown to exist in the peripheral terminals of capsaicin-sensitive sensory nerves (Burnstock, 1990; Lundberg, 1996). Although the origin of VIP-LI nerve fibres in peripheral tissues has not been established, there is much evidence that VIP-LI nerves in the spinal cord are markedly decreased by capsaicin treatment (Jancso *et al.*, 1981). Since these neuropeptides are known to be potent vasodilators (Burnstock, 1990; Lundberg, 1996), it is possible that active hyperaemia results from the release of CGRP, VIP and/or SP from capsaicin-sensitive nerves. The present study showed that a bolus intra-arterial injection of VIP or CGRP induced a relatively long-lasting increase in RBF of the rat hindlimb, which was similar to the response to sciatic nerve stimulation. We also found that the hyperaemic response to sciatic nerve stimulation was associated with a marked increase in serum CGRP-LI level, a slight increase in serum SP-LI level, but no appreciable increase in serum VIP-LI level. Although CGRP has been shown to coexist with acetylcholine in motor nerve terminals (Takami *et al.*, 1985), the origin of the elevated serum CGRP-LI is likely to be capsaicin-sensitive sensory nerves, but not motor nerves, because the increase in serum CGRP-LI level by sciatic nerve stimulation disappeared after treatment with capsaicin. These results led to the hypothesis that CGRP released from capsaicin-sensitive sensory nerves makes the greatest contribution to active hyperaemia in the rat hindlimb. Moreover, the ab-

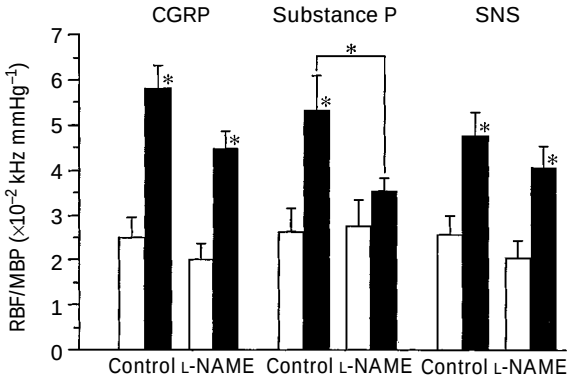

Figure 5 Effects of N_ω-nitro-L-arginine methyl ester (L-NAME) on hyperaemic responses to calcitonin gene-related peptide (CGRP; 300 pmol kg^{-1}, i.a.), substance P (SP; 200 pmol kg^{-1}, i.a.) and sciatic nerve stimulation (SNS; 10 Hz, 1 min). Approximately 20 min after control recordings, continuous infusion of L-NAME (1 μmol kg^{-1} min^{-1}, i.v.) was started. Hyperaemic responses to CGRP, SP or SNS were recorded before (Control) and 20 min after the start of L-NAME infusion. Open and solid columns represent values at the baseline and at the peak of the hyperaemic response, respectively. Data are expressed as mean ± s.e.mean of values obtained from six to nine rats. $^*P<0.05$ compared with baseline or respective control (ANOVA followed by the Bonferroni method).

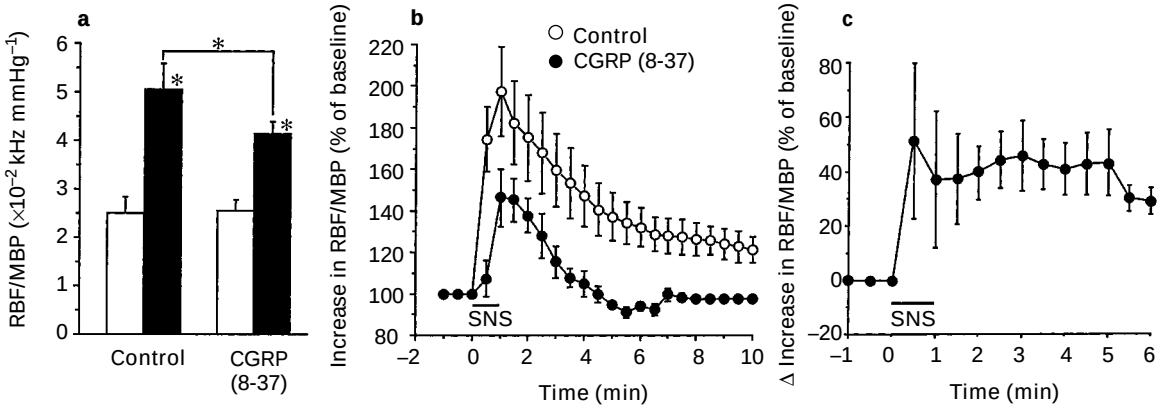

Figure 6 Effect of calcitonin gene-related peptide(8–37) (CGRP(8–37)) on the hyperaemic response to sciatic nerve stimulation (SNS). (a) Hyperaemic responses to SNS were recorded before (Control) and during continuous infusion of CGRP(8–37) (100 nmol kg^{-1} min^{-1}, i.v.). Open and solid columns represent values at the baseline and at the peak of the hyperaemic response, respectively. Data are expressed as the mean ± s.e.mean of values obtained from six to nine rats. $^*P<0.05$ compared with baseline or respective control (ANOVA followed by the Bonferroni method). (b) Effect of continuous infusion of CGRP(8–37) (100 nmol kg^{-1} min^{-1}, i.v.) on the time course of active hyperaemia. Vascular conductance was calculated every 30 s and expressed as % of basal vascular conductance. Note that CGRP(8–37) preferentially attenuated the sustained component of functional hyperaemia rather than the peak. Data are expressed as mean ($n=6$); vertical lines show s.e.mean. (c) Time course of CGRP(8–37)-sensitive hyperaemic response. The amplitude of the component sensitive to CGRP(8–37) was calculated by subtracting the response in the presence of CGRP(8–37) from the control in (b).

sence of an apparent change in serum CGRP-LI level after treatment with (+)-tubocurarine or capsaicin, which both largely suppressed the hyperaemic response to sciatic nerve stimulation, also supports the hypothesis. Finally, the $CGRP_1$ receptor antagonist, CGRP(8–37), significantly suppressed the sustained phase of the hyperaemic response to sciatic nerve stimulation. We thus concluded that CGRP is a plausible substance which is released from capsaicin-sensitive sensory nerves and mediates active hyperaemia. Other metabolic mediators proposed by a number of investigators (Haddy & Scott, 1975; Shephard, 1983), such as potassium, inorganic phosphate and adenosine, may take part in the transient response observed in the presence of CGRP(8–37). Although a slight increase in serum SP-LI level was also observed, the lack of an inhibitory effect of L-NAME on the hyperaemic response to sciatic nerve stimulation excludes the possibility of the involvement of SP in active hyperaemia under the present conditions. The response to SP was dependent on the release of NO to produce vasorelaxation, in agreement with previous work (Persson *et al*., 1991). Although CGRP often coexists with SP in the same population of neurones (Gibson *et al*., 1984; Lundberg *et al*., 1985), several immunohistochemical studies have shown that while most SP-positive neurones are CGRP-immunoreactive, cell bodies positive for CGRP but negative for SP are present in considerable numbers (Lundberg, 1996).

A number of studies have investigated the possible role of NO in active hyperaemia. Inhibition of NO synthase reduces exercise-induced vasodilatation in the rat hindquarter (Hirai *et al*., 1994) and human forearm (Gilligan *et al*., 1994). In contrast, NO synthase inhibitors have no effect on active hyperaemia in the rabbit tenuissimus muscle (Persson *et al*., 1990), the cat gastrocnemium muscle (Ekelund *et al*., 1992), and the human forearm (Wilson & Kapoor, 1993; Endo *et al*., 1994). In agreement with the latter studies, the present study showed that L-NAME had no effect on active hyperaemia in the rat hindlimb. As Haddy and Scott (1975) point out, it is very likely that the contribution of various factors to active hyperaemia changes with the duration and the level of exercise. Therefore, although it was concluded that NO is not associated with active hyperaemia brought about by 1 min, 10 Hz sciatic nerve stimulation in the rat hindlimb, the possibility that NO takes part in active hyperaemia under other conditions cannot be ruled out. Inconsistencies may also arise depending on the vascular bed studied and the animal species used.

From the findings obtained in the present study, the likely mechanism for active hyperaemia emerges as follows; the skeletal muscle contraction brings about excitation of neighbouring sensory nerves and, in turn, the release of CGRP from their peripheral endings around small blood vessels, thus leading to the hyperaemic response in the hindlimb. Immunohistochemical evidence for the extensive perivascular localization of sensory nerve fibres containing CGRP in the rat femoral artery and vein (Edvinsson *et al*. 1989) supports this hypothesis. The excitation of capsaicin-sensitive nerves may be propagated to neighbouring areas via axon reflex-like mechanisms and release CGRP from the peripheral endings of the sensory nerves (Holzer, 1988; Lundberg, 1996). Some experimental evidence favouring these arguments has been obtained. For instance, guinea-pig mesenteric arteries receive capsaicin-sensitive sensory innervation from the distal colon, and the sensory nerves are activated during periods of colonic distention, i.e., a mechanism stimulus, which induces hyperpolarization of arterial smooth muscle (Meehan & Kreulen, 1992). Metabolic stimuli such as low pH also appear to induce the release of CGRP from capsaicin-sensitive sensory nerves in the rat stomach (Geppetti *et al*., 1991b) and soleus muscle (Santicioli *et al*., 1992) and the guinea-pig heart (Franco-Cereceda *et al*., 1993) and urinary bladder (Geppetti *et al*., 1991a).

In summary, the present study showed that contraction of the rat hindlimb induced by sciatic nerve stimulation evokes active hyperaemia in part via neuronal mechanisms. The data provide the first evidence of a contribution of peripheral sensory nerves to active hyperaemia. We propose that CGRP released from sensory nerve endings is one of the most feasible endogenous substances mediating active hyperaemia in the rat hindlimb.

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