British Journal of Pharmacology (1997) 122, 710–714

Local neurogenic regulation of rat hindlimb circulation: CO_2-induced release of calcitonin gene-related peptide from sensory nerves

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1 The mechanism of release of calcitonin gene-related peptide (CGRP) from sensory nerves in response to skeletal muscle contraction was investigated in the rat hindlimb *in vivo* and *in vitro*.

2 In the anaesthetized rat, sciatic nerve stimulation at 10 Hz for 1 min caused a hyperaemic response in the hindlimb. During the response, partial pressure of CO_2 in the venous blood effluent from the hindlimb significantly increased from 43 ± 3 to 73 ± 8 mmHg, whereas a small decrease in pH and no appreciable change in partial pressure of O_2 were observed.

3 An intra-arterial bolus injection of $NaHCO_3$ (titrated to pH 7.2 with HCl), which elevated P_{CO_2} of the venous blood, caused a sustained increase in regional blood flow of the iliac artery. Capsaicin (0.33 μmol kg^{-1}, i.a.) and a specific calcitonin gene-related peptide (CGRP) receptor antagonist, CGRP(8–37), (100 nmol kg^{-1} min^{-1}, i.v.) significantly suppressed the hyperaemic response to $NaHCO_3$. Neither ND_ω-nitro-L-arginine methyl ester (1 μmol kg^{-1} min^{-1}, i.v.) nor indomethacin (5 mg kg^{-1}, i.v.) affected the response.

4 The serum level of CGRP-like immunoreactivity in the venous blood was significantly increased by a bolus injection of $NaHCO_3$ (pH = 7.2) from 50 ± 4 to 196 ± 16 fmol ml^{-1}.

5 In the isolated hindlimb perfused with Krebs-Ringer solution, a bolus injection of $NaHCO_3$ (pH = 7.2) caused a decrease in perfusion pressure which was composed of two responses, i.e., an initial transient response and a slowly-developing long-lasting one. CGRP(8–37) significantly inhibited the latter response by 73%.

6 These results suggest that CO_2 liberated from exercising skeletal muscle activates capsaicin-sensitive perivascular sensory nerves locally, which results in the release of CGRP from their peripheral endings, and then the released peptide causes local vasodilatation.

Keywords: CO_2; calcitonin gene-related peptide; sensory nerve; skeletal muscle; hyperaemia

Introduction

Recent progress in immunohistochemistry has revealed the presence of a variety of peptides in afferent sensory nerves in peripheral tissues (Holzer, 1988; Burnstock, 1990; Lundberg, 1996). These peptides, e.g., calcitonin gene-related peptide (CGRP) and substance P, are mostly contained in the peripheral endings of sensory nerves, especially dense around blood vessels, and may therefore play a neurotransmitter or neuromodulator role in peripheral tissues (Holzer, 1988; Burnstock, 1990; Lundberg, 1996). CGRP is a potent vasodilator in a variety of vascular beds of different species (Bell & McDermott, 1996), including man (Franco-Cereceda *et al*., 1987; Miyauchi *et al*., 1996). CGRP-induced vasodilatation is mostly endothelium-independent and typically long-lasting (Bell & McDermott, 1996; Lundberg, 1996). Increasing evidence suggests that CGRP plays a role as a neurotransmitter which mediates sensory nerve-evoked vasodilatation. For example, the vasodilatation evoked by electrical antidromic nerve stimulation is markedly reduced by CGRP(8–37) in rat skin (Delay-Goyet *et al*., 1992) and dental pulp and lip (Kerezoudis *et al*., 1994). However, the physiological role of CGRP in peripheral tissues remains to be elucidated fully.

In our previous work (Yamada *et al*., 1997), we showed that CGRP is released from peripheral endings of sensory nerves after hindlimb muscle contraction and causes local vasodilatation. Thus, the present study was designed to elucidate the mechanism of CGRP release from sensory nerves after skeletal muscle contraction.

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Methods

In vivo *experiments*

Male Wistar rats (260–350 g; Charles River, Kanagawa, Japan) were anaesthetized with urethane (1.5 g kg^{-1}, i.p.) and placed on a water-perfused heating pad set at 37°C to maintain a constant body temperature. Systemic 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 in 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 left iliac artery and connected to a pulsed Doppler velocimeter (Crystal Biotech PD-20, Holliston, MA, U.S.A.) to allow continuous recording of regional blood flow (RBF) of the left hindlimb as a shift in kHz of Doppler signals. All experiments were performed after treatment with propranolol (1 mg kg^{-1}, i.v.) for reasons described previously (Yamada *et al*., 1997). We compared values of RBF/MBP at the baseline and the peak of hyperaemia, which reflects changes in tension of the resistance vessels.

Changes in pH, partial pressure of CO_2 (P_{CO_2}) and partial pressure of O_2 (P_{O_2}) of the venous blood were measured as follows. About 0.2 ml of venous blood was collected via a catheter inserted into the left iliac vein before and 1 min after the cessation of sciatic nerve stimulation (SNS) or administration of $NaHCO_3$. In some experiments, the same volume of arterial blood was collected through a catheter inserted in the

right iliac artery. Immediately the blood had been collected, pH, P_{CO_2} and P_{O_2} were measured by an automatic pH/blood gas analyser (Ciba Corning 238, Tokyo, Japan). No significant change in the variables was observed in the second collection of blood without any treatment.

Serum levels of CGRP were measured by radioimmunoassay (RIA). A catheter was inserted into the left iliac vein. About 0.35 ml of venous blood was collected 1 min after injection of $NaHCO_3$. The collected blood was mixed with aprotinin (300 iu ml^{-1}) and EDTA-2Na (2 mg ml^{-1}) and centrifuged immediately (2000 × g, 15 min), the serum obtained was stored at −80°C until assay. Only one blood sample was taken from each rat. Serum CGRP-like immunoreactivity (CGRP-LI) was determined by RIA according to the method described previously (Fujimori *et al*., 1989). Rabbit antiserum against rat α-CGRP was purchased from Peninsula (Belmont, CA, U.S.A.).

In vitro *experiments*

Isolated, perfused hindlimb preparation was prepared according to the method of Ahokas and Sibai (1992) with minor modification. Briefly, male Wistar rats (350–400 g; Charles River, Kanagawa, Japan) were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.). Catheters were inserted into the iliac artery just distal to the abdominal aorta bifurcation and advanced into the femoral arteries. The hindlimb was gently flushed with 0.9% saline solution containing heparin (100 u ml^{-1}). After the rat was killed by administration of excess anaesthetic agent through the catheter inserted in the carotid artery, the hindlimb was sectioned from the body and placed in a water-jacketed chamber, maintained at 37°C, and perfused at a constant flow rate of 4.0 ml min^{-1} with Krebs-Ringer solution by a peristaltic pump (Minipuls 3, Gilson, Bel, France). The Krebs-Ringer solution had the following composition (mM): NaCl 113, KCl 4.8, $NaHCO_3$ 25, $CaCl_2$ 2.2, $MgSO_4$ 1.2, KH_2PO_4 1.2, and glucose 5.5. The solution was maintained at 37°C and aerated with a mixture of 95% O_2 and 5% CO_2. After a 30 min perfusion period to wash out blood and allow perfusion pressure to stabilize, perfusion pressure was measured with a pressure transducer (SCK-590, Gould, Cleveland, OH, U.S.A.). Throughout the experiment, 10 μM indomethacin and 100 μM N_ω-nitro-L-arginine methyl ester (L-NAME) were contained in the perfusing Krebs-Ringer solution.

Statistics

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

Drugs

Drugs used were (+)-tubocurarine chloride (Wako Chemicals, Osaka, Japan); rat α-calcitonin gene-related peptide (CGRP), human α-CGRP(8–37) (Peptide Institute, Osaka, Japan); heparin sodium (Novo Nordisc A/S, Denmark); capsaicin, N_ω-nitro-L-arginine methyl ester (L-NAME), indomethacin, propranolol (Sigma, St. Louis, MO, U.S.A.); and urethane (Carbamic acid ethylester; Tokyo Kasei, Tokyo, Japan).

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. Indomethacin was dissolved in dimethyl sulphoxide. Other drugs were dissolved in saline or water. $NaHCO_3$ was dissolved in distilled water at a concentration of 0.6 M. Just before administration the $NaHCO_3$ solution was titrated to pH 7.2 with 2 N HCl.

Results

Changes in chemical variables in venous blood during functional hyperaemia

Electrical stimulation of the sciatic nerve at 10 Hz for 1 min caused a marked increase in RBF of the iliac artery, which remained above the basal level for more than 5 min after the cessation of nerve stimulation (data not shown). Under this condition, P_{CO_2} of venous blood effluent from the hindlimb markedly increased during the hyperaemic response after skeletal muscle contraction, whereas only a slight decrease in pH and no appreciable change in P_{O_2} were observed (Figure 1). None of these variables in arterial blood collected from the iliac artery was affected by sciatic nerve stimulation (data not shown).

Effects of $NaHCO_3$ on hindlimb circulation in vivo

To produce an increase in P_{CO_2} without changes in P_{O_2} and pH of the venous blood, we prepared a $NaHCO_3$-containing solution titrated to pH 7.2 with HCl. Intra-arterial bolus injection of $NaHCO_3$ (0.6 mmol kg^{-1}, i.a.) caused changes in the chemical factors of venous blood effluent similar to those induced by sciatic nerve stimulation; i.e., a significant increase in P_{CO_2} and no significant change in pH or P_{O_2} (Figure 1). As illustrated in Figure 2, a bolus injection of $NaHCO_3$ produced a long-lasting increase in RBF of the iliac artery. Since it has been proposed that $NaHCO_3$-induced hyperaemia is due to an

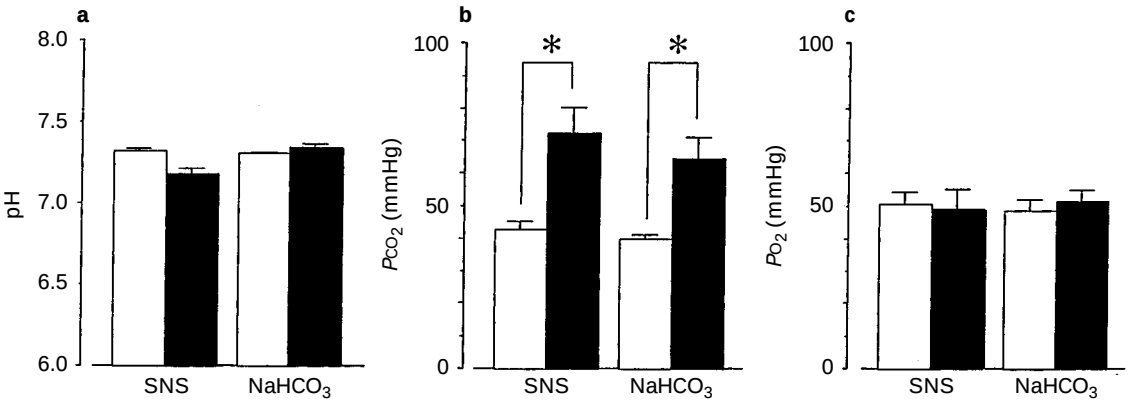

Figure 1 Changes in (a) pH, (b) partial pressure of CO_2 (P_{CO_2}) and (c) partial pressure of O_2 (P_{O_2}) of venous blood by sciatic nerve stimulation (SNS) or injection of $NaHCO_3$ (0.6 mmol kg^{-1}, pH = 7.2). Values of pH, P_{CO_2} and P_{O_2} of venous blood were measured by an automatic pH/blood gas analyser. Open and solid columns represent values before and 1 min after SNS or injection of $NaHCO_3$, respectively. Data are expressed as mean ± s.e.mean of values obtained from six rats. *$P < 0.05$ versus control (Student's t test for paired data).

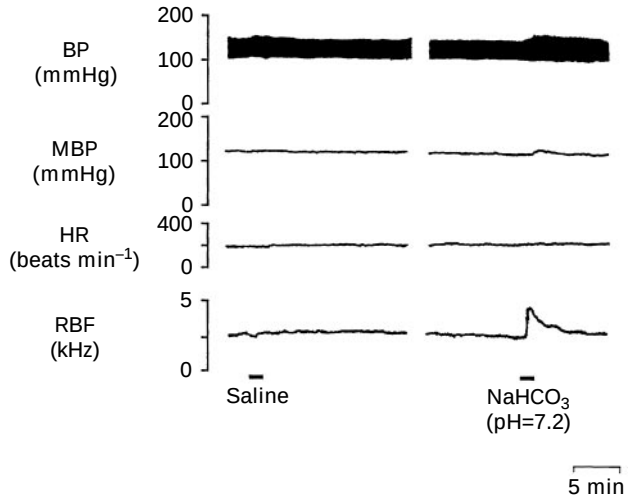

Figure 2 Typical traces of the effect of intra-arterial injection of $NaHCO_3$ (0.6 mmol kg^{-1}, pH = 7.2) on systemic blood pressure (BP), mean blood pressure (MBP), heart rate (HR), and regional blood flow of the iliac artery (RBF).

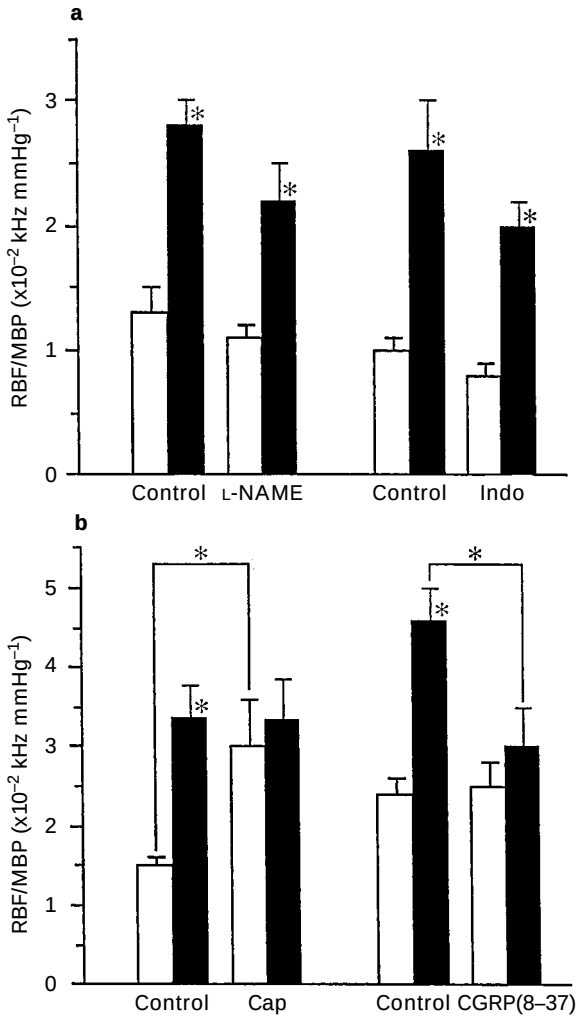

Figure 3 Effects of various drugs on the hyperaemic response to $NaHCO_3$ *in vivo*. The increase in RBF/MBP induced by intra-arterial injection of $NaHCO_3$ (0.6 mmol kg^{-1}, pH = 7.2) before (Control) and after treatment with N_ω-nitro-L-arginine methyl ester (L-NAME; 300 μg kg^{-1} min^{-1}, i.v.), indomethacin (Indo; 5 mg kg^{-1}, i.v.), capsaicin (Cap; 100 μg kg^{-1}, i.a.) or calcitonin gene-related peptide(8–37) (CGRP(8–37); 100 nmol kg^{-1} min^{-1}, i.v.) were compared. 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 animals. $*P < 0.05$ compared with baseline or respective control (ANOVA followed by the Bonferroni method).

increase in osmotic pressure rather than a P_{CO_2} in the monkey cerebral artery (Toda *et al*., 1993), we tested the effects of NaCl solution with comparable osmolality to that of the $NaHCO_3$ solution. Intra-arterial injection of NaCl solution caused only a slight, transient increase in RBF, which was less than 15% of the response to $NaHCO_3$ ($n = 3$, data not shown). We thus concluded that most of the hyperaemic response to $NaHCO_3$ was independent of osmotic pressure in the rat hindlimb.

In cerebral arteries, CO_2-induced vasodilatation is mediated by factors released from endothelial cells, i.e., nitric oxide (NO) (Iadecola, 1992) and vasodilator prostaglandins (Wagerle & Mishra, 1988; Hsu *et al*., 1993). We therefore examined the possible involvement of NO or prostaglandins in $NaHCO_3$-induced hyperaemia in the rat hindlimb. As shown in Figure 3a, the hyperaemia induced by $NaHCO_3$ (0.6 mmol kg^{-1}, i.a.) was not affected by continuous infusion of a NO synthase inhibitor, L-NAME (300 mg kg^{-1} min^{-1}, i.v.), or bolus injection of a cyclo-oxygenase inhibitor, indomethacin (5 mg kg^{-1}, i.v.). These concentrations of L-NAME and indomethacin almost abolish the hyperaemic response to substance P (Yamada *et al*., 1997) and the depressor effect of arachidonic acid (Miyauchi *et al*., 1989), respectively. It was therefore suggested that neither NO nor a prostaglandin is involved in $NaHCO_3$-induced hyperaemia in the rat hindlimb.

Effect of $NaHCO_3$ on capsaicin-sensitive sensory nerves in vivo

To elucidate whether $NaHCO_3$ releases CGRP from peripheral endings of capsaicin-sensitive sensory nerves, we investigated the effects of capsaicin on the hyperaemic response to $NaHCO_3$. Capsaicin is now widely used as a specific probe for investigating the function of sensory nerves. At relatively low doses, capsaicin exerts a powerful excitatory effect on peripheral sensory nerve endings, which is soon followed by a block of nerve conduction, whereas administration of high doses has a definite neurotoxic effect on sensory neurones (Holzer, 1991;

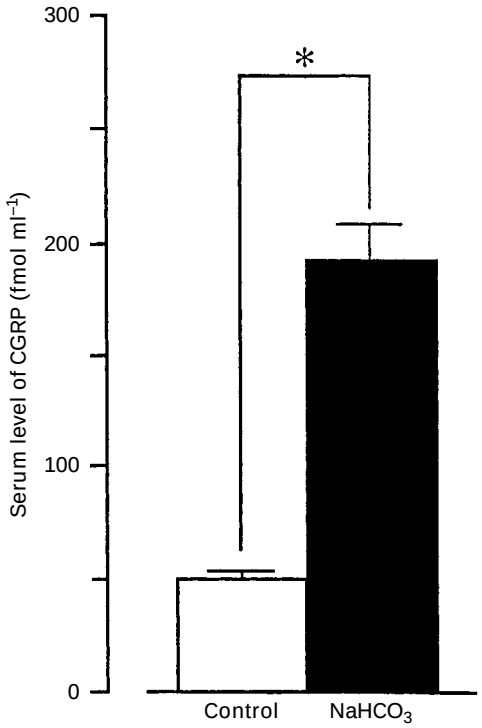

Figure 4 Change in serum level of CGRP-like immunoreactivity (LI) in venous effluent induced by injection of $NaHCO_3$ (0.6 mmol kg^{-1}, pH = 7.2). The serum level of CGRP-LI was measured by radioimmunoassay. Data are expressed as mean ± s.e.mean of values obtained from 10 animals for each column. $*P < 0.05$ versus control (Student's t test for unpaired data).

Maggi, 1991a). A bolus injection of capsaicin (100 μg kg^{-1}, i.a.) produced a marked increase in RBF, which reached the maximal level in 40–50 min, then gradually decreased, and reached a plateau level, which was still significantly higher than the basal level in 70–90 min (Figure 3b). We found that $NaHCO_3$ (0.6 mmol kg^{-1}, i.a.) did not cause a significant increase in RBF/MBP 90 min after the injection of capsaicin (Figure 3b). Since neither reactive hyperaemia nor exogenously applied CGRP was affected by capsaicin treatment (Yamada *et al*., 1997), the decline in the $NaHCO_3$-induced response is unlikely to result from the increased basal level caused by capsaicin. We next examined the effect of a CGRP receptor antagonist, CGRP(8–37), on $NaHCO_3$-induced hyperaemia. Continuous infusion of CGRP(8–37) (100 nmol kg^{-1} min^{-1}, i.v.) almost abolished the increase in vascular conductance induced by bolus injection of $NaHCO_3$ (0.6 mmol kg^{-1}, i.a.) (Figure 3b).

We further assessed changes in the serum concentration of CGRP-LI in venous effluent by use of RIA. As shown in Figure 4, serum CGRP-LI level in venous blood was significantly increased by intra-arterial injection of $NaHCO_3$ (0.6 mmol kg^{-1}), indicating that CGRP is actually released during the hyperaemic response.

Effects of $NaHCO_3$ on perfusion pressure in isolated hindlimb

In the perfused hindlimb with active tone produced by continuous perfusion of 10 μM methoxaime in the presence of 10 μM indomethacin and 100 μM L-NAME, a bolus injection of $NaHCO_3$ (pH = 7.2, 160 μmol) produced a marked decrease in perfusion pressure. The vasodilatation was composed of two responses; an initial transient response and a slowly-developing long-lasting one (Figure 5). The latter lasted more than 5 min. When CGRP(8–37) (4 nmol) was injected as a bolus 2 min before the injection of $NaHCO_3$, the long-lasting response to $NaHCO_3$ was significantly attenuated, while the transient one was not affected (Figure 5), suggesting that the slowly-developing long-lasting response to $NaHCO_3$ is mediated by CGRP. When NaCl solution the osmolality of which was comparable to that of the $NaHCO_3$ solution was injected, only a transient decrease in perfusion pressure was observed (data not shown), which was similar to the transient response to $NaHCO_3$. It is therefore likely that the transient response to $NaHCO_3$ was produced by a change in osmolality.

Discussion

Our preceding paper showed that CGRP released from capsaicin-sensitive sensory nerves causes neurogenic active hyperaemia after a short period of skeletal muscle contraction (Yamada *et al*., 1997). In the present study, we further explored the mechanism of CGRP release from sensory nerves after skeletal muscle contraction. Since CGRP is contained in the peripheral endings of sensory nerves (Burnstock, 1990; Lundberg, 1996), it allows one to hypothesize that some factor(s) appearing during skeletal muscle contraction may stimulate sensory nerves and release CGRP from their peripheral endings. The present study showed for the first time that

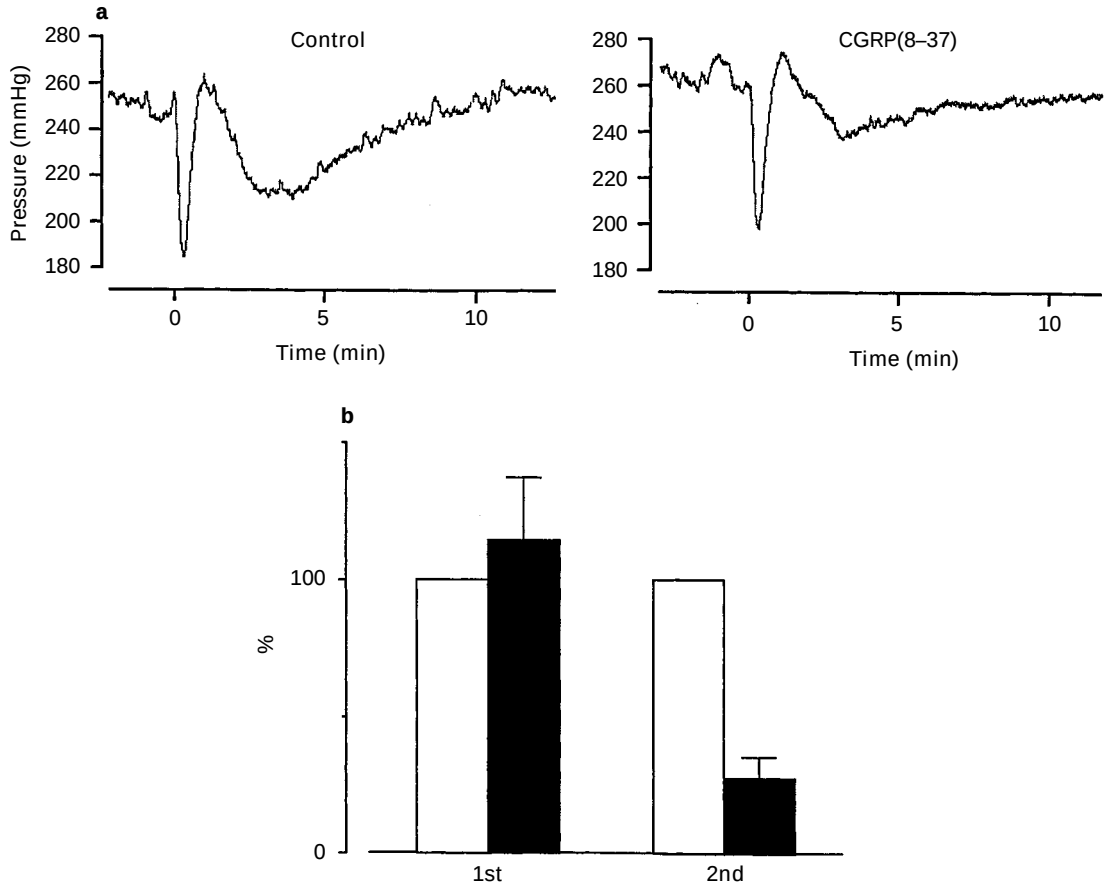

Figure 5 Effect of $NaHCO_3$ on perfusion pressure in isolated perfused hindlimb. (a) Typical traces of vasodilator response to $NaHCO_3$ (160 μmol, pH = 7.2) before (left panel) and after (right panel) treatment with calcitonin gene-related peptide(8–37) (CGRP(8–37); 4 nmol). $NaHCO_3$ was administered at time 0. Note that the response to $NaHCO_3$ is composed of two phases. (b) Mean vasodilator responses to $NaHCO_3$ (160 μmol, pH = 7.2) after treatment with CGRP(8–37) (4 nmol). The first transient phase of the response (1st) and the second slowly-developing long-lasting phase of the response (2nd) after treatment with CGRP(8–37) are expressed as % of corresponding responses before treatment. Open and solid columns represent values before and after treatment with CGRP(8–37) respectively. Data are expressed as mean ± s.e.mean of values obtained from five preparations.

$NaHCO_3$ releases CGRP, most probably mediated by CO_2, by activating capsaicin-sensitive sensory nerves.

It has been shown that CGRP is released from sensory nerves by some exogenous or endogenous substances, including capsaicin, bradykinin, nicotine, histamine, ouabain, arachidonic acid and prostaglandins, and by conditions such as low-pH and high-K^+ in various experimental models (Maggi, 1991b; Lundberg, 1996). Santicioli *et al*. (1992) have demonstrated that the rat soleus muscle receives sensory innervation by capsaicin-sensitive primary afferents which release CGRP-LI in response to low-pH (pH = 6 or 5) media. Electrophysiological evidence suggests that protons (pH <6.2) open a cation channel similar to that opened by capsaicin in a subpopulation of rat dorsal root ganglion neurones (Bevan & Yeats, 1991). Since the pH of plasma is controlled strictly and adjusted rapidly by renal and respiratory mechanisms, it is unlikely that sensory nerve fibres are stimulated by the small changes in systemic pH. However, in skeletal muscle, intense muscular exercise can cause the intramuscular pH to fall to levels below 6.5 (Victor *et al*., 1988). Therefore, during prolonged exhaustive exercise, the local pericellular low-pH may act together with CO_2 to activate capsaicin-sensitive sensory nerves and release CGRP

It appears that capsaicin-sensitive primary sensory nerves release transmitters not only from their central endings but also at the periphery, thus having a dual sensory and efferent function (Holzer, 1988; Lundberg, 1996). The present study showed that a hyperaemic response to $NaHCO_3$ was produced in the isolated, perfused hindlimb *in vitro*. This finding suggests that the response occurs locally, not systemically. One possible mechanism for the response is that the CO_2-evoked release of CGRP from sensory nerves in the hindlimb is mediated by the 'axon reflex' (Holzer, 1990; Lundberg, 1996). Alteratively, it is possible that the release of CGRP is produced via direct activation by CO_2 of the nerve endings without an obligatory contribution of propagated nerve activity, as produced by capsaicin, nicotine and low pH (Holzer, 1991; Maggi, 1991a; Lundberg, 1996). Further investigation will be needed to determine the mode of CGRP release from sensory nerves by CO_2.

In summary, the present study showed that $NaHCO_3$ causes release of CGRP, most probably mediated by CO_2, from capsaicin-sensitive sensory nerves in the rat hindlimb. Taken together with our previous finding that CGRP partly mediates active hyperaemia (Yamada *et al*., 1997), we propose a novel mechanism of active hyperaemia involving CO_2 and sensory 'efferent' nerves in the rat hindlimb: CO_2 liberated from exercising skeletal muscle stimulates capsaicin-sensitive sensory nerves. The excitation of sensory nerves then releases the vasodilator peptide CGRP from their peripheral endings and thereby active hyperaemia is produced.

This study was supported by a grant of the Special Research Project on the Circulation Biosystem from the University of Tsukuba and a grant-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan. We would like to thank Dr W.A. Gray for language editing.

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(Received November 5, 1996
Revised June 23, 1997
Accepted July 7, 1997)