

Frequency Dependence of Local Cerebral Blood Flow Induced by Somatosensory Hind Paw Stimulation in Rat under Normo- and Hypercapnia

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Abstract: We measured the field potential and the changes in local cerebral blood flow (LCBF) response during somatosensory activation (evoked LCBF) in α -chloralose-anesthetized rats by laser-Doppler flowmetry under normocapnia ($P_{aCO_2}=34.3\pm 3.8$ mmHg) and hypercapnia ($P_{aCO_2}=70.1\pm 9.8$ mmHg). Somatosensory activation was induced by electrical stimulation (0.2, 1, and 5 Hz with 1.5 mA for 5 s) of the hind paw. The neuronal activity of the somatosensory area of the hind paw was linear to the stimulus frequency, and there was no significant difference in the neuronal activity between hypercapnia and normocapnia. The baseline level of LCBF under hypercapnia was about 72.2% higher than that under normocapnia ($p<0.01$). The absolute response magnitude under hypercapnia was greater than that under normocapnia ($p<0.05$). The evoked LCBF under both conditions showed a frequency-dependent increase in the 0.2 to 5 Hz range, and the difference in the absolute re-

sponse magnitude at the same stimulus frequency between normocapnia and hypercapnia became large with increasing stimulus frequency ($p<0.05$). On the other hand, after normalization to each baseline level there was no significant difference in the response magnitude of the normalized evoked LCBF between normocapnia and hypercapnia, indicating that the normalized evoked LCBF reflects neuronal activity even when the baseline LCBF was changed by the P_{aCO_2} level. The peak time and termination time of LCBF response curves with respect to the graded neuronal activity at 1 and 5 Hz stimulation increased significantly under hypercapnia, compared with those under normocapnia ($p<0.05$), although the rise time of 0.5 s was nearly constant. In conclusion, the results suggest a synergistic effect of the combined application of graded neuronal stimuli and hypercapnia on the LCBF response. [Japanese Journal of Physiology, 51, 201–208, 2001]

Key words: cerebral blood flow, hind paw stimulation, hypercapnia, somatosensory cortex, laser-Doppler flowmetry.

A fundamental aspect of brain physiology is the coupling between regional neuronal activation and changes in the local cerebral blood flow (LCBF). It has been suggested that the coupling process (1) is highly complicated and includes the combined action of many biochemical mediators (e.g., K^+ , H^+ , Ca^{2+} , adenosine, and nitric oxide) [1–4], and (2) is highly

localized; for example, somatosensory, visual, or auditory stimulation alters LCBF in discrete and appropriate brain regions [3, 5–7]. The abundance of nerve terminals at the vascular wall also suggests that functional activation is under neurogenic control [8]. However, despite much research over several decades, the exact mechanism underlying the coupling between

Received on September 20, 2000; accepted on January 25, 2001

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neuronal activity and LCBF response still remains to be elucidated.

Alterations in the $PaCO_2$ level have a marked effect on the cerebrovascular tone. This was expressed as an exponential relationship between LCBF and $PaCO_2$ with a baseline flow increase of about 2 to 5% per mmHg $PaCO_2$ [7, 9–12]. This supposes that an increase in LCBF during neuronal activity (evoked LCBF) may be affected by changes in the baseline flow that are induced by changes in the $PaCO_2$ level, in spite of a constant level of neuronal activity. Following a 13-s stimulation of rat barrel cortex, Gerrits *et al.* reported that no significant difference was noted in the frequency-dependent linear increase in the evoked LCBF under normocapnia and hypercapnia [11]. They also reported an initial peak followed by a plateau during long stimulation under normocapnia, but this initial peak disappeared under hypercapnia [11]. On the other hand, the relationship between the evoked LCBF induced by graded stimulus frequencies of short periods, which disrupt the biphasic response and leave only the early transient reaction of evoked LCBF under normocapnia, and changes in the $PaCO_2$ level have not been clarified. Moreover, to our knowledge there was no investigation of the effect of hypercapnia on the relationship between graded neuronal activity and absolute response of the evoked LCBF.

Laser-Doppler flowmetry (LDF) is a well-established technique for the continuous monitoring of LCBF, with time resolution in the millisecond range, and it yields flow data from a depth of approximately 1 mm below the cortical surface [9, 13–16]. Therefore LDF has been widely used to determine the characteristics of activation flow coupling response in animal models under normal and abnormal conditions. It is possible, using conventional microelectrode techniques and LDF, to determine the exact spatial and temporal relationship between the evoked nerve activity in the cortex and LCBF [14]. In the present study, using recordings of changes in electrical and LDF signals of the somatosensory cortex during stimulation of the hind paw, we investigated the changes in the neuronal activity and evoked LCBF response with respect to variations in the frequency of a short stimulation period (5 s) under normocapnia and hypercapnia.

MATERIALS AND METHODS

Animal preparation. All experiments were conducted in accordance with the guidelines of the Physiological Society of Japan and were approved by the Animal Care and Use Committee of the Research Institute for Brain and Blood Vessels, Akita, Japan.

Sprague-Dawley rats (385.0 ± 36.5 , $n=21$, mean \pm SD) were anesthetized with halothane (3% for induction and 1% during surgery) in 30% O_2 and 70% N_2O , using a face mask. Lidocaine (2%) was subcutaneously applied before incision to prevent vasospasm during catheter insertion. Polyethylene catheters were used to cannulate the tail artery and the left femoral vein for blood pressure monitoring, blood sampling for gas analysis, and intravenous (I.V.) administration of an anesthetic. After tracheotomy, α -chloralose (56 mg/kg, I.V.) was administered, and halothane and nitrous oxide administration was discontinued. Anesthesia was maintained with α -chloralose (44 mg/kg/h, I.V.) and muscle relaxation with pancronium bromide (0.7 mg/kg/h, I.V.). The body temperature was monitored with a rectal probe and maintained at about 37.0°C, using a heating pad (MK-900, Muromachi Kikai Co., Ltd., Japan).

The rat was ventilated by using a respirator (M-683, Harvard Apparatus, USA) throughout the experimental period with a mixture of air and oxygen to achieve physiological arterial blood levels of O_2 and CO_2 tension (PaO_2 and $PaCO_2$, respectively). Under normocapnia, $PaCO_2$ levels were maintained in the range of 35 to 40 mmHg and PaO_2 levels in the range of 110 to 130 mmHg by regulating the stroke volume of ventilation and the fractional concentration of oxygen in the gas inspired, respectively.

The rat was fixed in a stereotactic frame, and the parietal bone over the left somatosensory cortex was thinned to translucency using a dental drill (an area of 3×3 mm², centered at 2.5 mm caudal and 2.5 mm lateral to the bregma). To ensure a stable physiological condition of the animal, measurements were performed 3 h after the preparation of the parietal bone.

Hind paw stimulation and LCBF measurement. Electrical hind paw stimulation was performed with two needle electrodes inserted subdermally into the right hind paw contralateral to the LDF probe. For the analysis of frequency dependence, a current stimulus of 1.5 mA (0.1 ms pulse) was applied at a frequency of 0.2, 1, and 5 Hz with a duration of 5 s. The order of stimulus frequencies was selected randomly; at each stimulus frequency, 20 successive stimuli were applied at 60 s intervals (Fig. 1). These stimulus parameters caused no changes in the systemic arterial blood pressure and heart rate during stimulation [16, 17].

Changes in LCBF were measured by LDF (TDF-LN1, Unique Medical, Japan). The tip diameter of the LDF probe was 0.55 mm (Probe LP-N, Unique Medical). LDF measures the behavior of red blood cells in the capillary based on the Doppler effect with laser

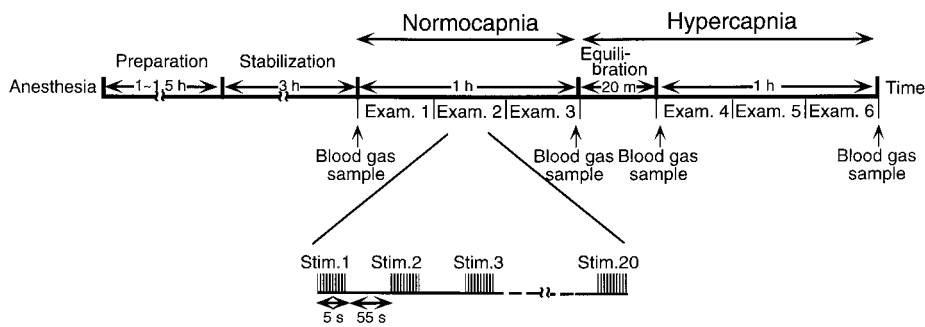


Fig. 1. Protocol of experiment. Examination was carried out about 3 h after preparation of the animals. The LCBF responses under normocapnia were initially examined, and then those under hypercapnia, 20 min after 2.5% CO₂ inhalation. At each examination, 20 successive stimuli of 0.2, 1, and 5 Hz frequency with a duration of 5 s were applied at 60 s intervals. The order of stimulus frequencies was selected randomly.

light (wavelength of 780 nm). The frequency shift of scattered radiation is caused by moving red blood cells in the blood vessels. The sampling volume of LDF measurement was about 1 mm³ [18]. A time constant of 0.1 s was used to detect the LDF signal. The LDF probe was positioned on the thinned skull (over the somatosensory area of the hind paw) perpendicular to the brain surface. It was attached to the thinned parietal bone, then finely positioned by using a micro-manipulator to obtain the maximum signal change during stimulation (15 to 20% at a frequency of 5 Hz, current of 1.5 mA, and duration of 5 s), avoiding areas with large blood vessels.

We examined the LCBF response under normocapnia, followed by that under hypercapnia after 20 min of hypercapnic ventilation ($n=12$) (Fig. 1). For hypercapnic ventilation, approximately 2.5% CO₂ was mixed with the gas administered under normocapnia. An equilibration time of 20 min was the time at which the baseline LCBF reached a peak value after 2.5% CO₂ ventilation [12]. LDF signals were recorded continuously by using MacLab data acquisition software (AD Instruments, Australia), and the outputs were accumulated 20 times. We have confirmed that our experimental schedule did not affect the physiological condition, baseline LCBF, and evoked LCBF (data not shown).

Field potential measurement. To confirm the induction of neuronal activity during the somatosensory stimulations, the field potentials under normocapnia and hypercapnia were recorded in the other 9 rats. A tungsten microelectrode (12 M Ω) was inserted into the somatosensory area of the hind paw through the thinned portion of the skull and was fixed with dental cement. The tip of the electrode was set at a depth of about 0.5 mm from the surface of the cortex. An Ag–AgCl indifferent electrode was placed between the skull bone and the scalp. At first we recorded the field potentials under the normocapnia, then those under the hypercapnia following the same

time schedule as that for the LCBF measurement. Twenty successive signals of the field potential recordings were also accumulated by using the MacLab data acquisition software. In the field potential analysis, the number of spike-shaped potentials that exceeded the noise level during the stimulation period was counted. The mean amplitude of the field potentials was also calculated as the average of the positive components of each potential.

Data analysis. Arterial blood pressure was monitored during the experiments, and the mean arterial blood pressure (MABP) was calculated as the average at three time points (i.e., before, during, and immediately after each examination). Arterial blood samples were serially collected before and immediately after each step of examination and analyzed for blood gas values (Fig. 1).

The LDF data were digitized at 40 Hz and saved on a disk for off-line analysis. The rise time and the termination time of the evoked LCBF were defined as the time points at the intersection of the extrapolated lines, which were drawn on the response curve from 90 to 10% of the peak, with the baseline [16]. The peak time was the time at which the response curve of LCBF reached the maximum height. The response magnitudes of evoked LCBF were precalculated by using the following two methods (Fig. 2):

(1) Subtraction of the baseline level: In the present paper we call these values “absolute response magnitude,” which was calculated as an integral above the baseline level of the raw response curve (absolute LDF value) from the rise time to the termination time.

(2) Normalization to the baseline level: In the present paper we call these values “normalized response magnitude,” which was calculated as an integral of the normalized response curve (time course of percent change from baseline value) from the rise time to the termination time.

In general, LDF data are normalized to a certain criterion. Therefore we normalized the absolute re-

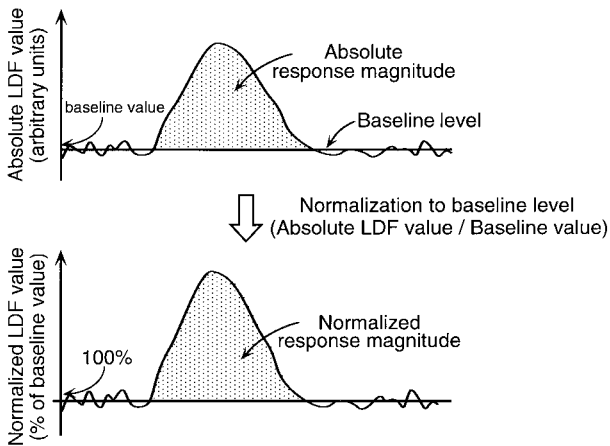


Fig. 2. Schematic diagram illustrating the calculation method of absolute and normalized response magnitude of evoked LCBF. The absolute response magnitude was calculated as the integral above the baseline level of the raw response curve (absolute LDF value) from the rise time to the termination time. The normalized response curves of LDF signals, which indicate time courses of percent changes from the baseline level, were calculated by dividing raw data by each baseline values, and the normalized response magnitude was the integral of the normalized response curve from the rise time to the termination time.

Table 1. Physiological variables.

Parameter	Normocapnia	Hypercapnia	<i>p</i>
MABP	101.0±13.6	85.4±10.5	<0.05
pH	7.4±0.1	7.1±0.1	<0.01
PaCO ₂	34.3±3.8	70.1±9.8	<0.01
PaO ₂	121.6±18.0	125.3±16.2	ns

Data include both groups of LCBF and field potential measurements. Mean±SD (*n*=21).

response magnitudes to that at 0.2 Hz stimulation under normocapnia after statistical analysis. LDF and electrophysiological data were statistically analyzed by using ANOVA and a Student's *t*-test or Bonferroni test. Values are presented as means±SD.

RESULTS

Effect of hypercapnia on physiological variables

As shown in Table 1, the physiological variables were within the normal ranges throughout the experimental period under normocapnia. Ventilation with 2.5% CO₂ for 20 min caused a significant reduction in arterial pH, a significant elevation in the level of PaCO₂ and a significant decrease in MABP (*p*<0.05) (Table 1). PaO₂ levels were unaffected by CO₂ inhala-

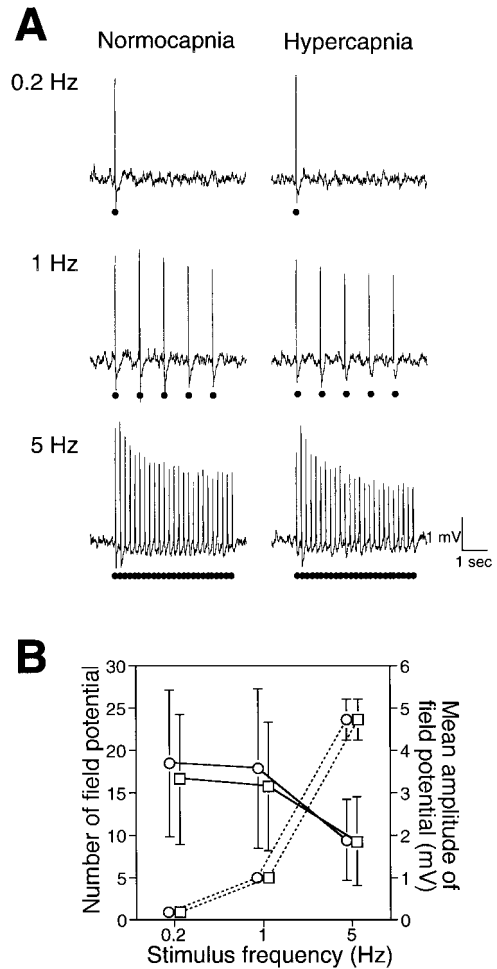


Fig. 3. Neuronal activity at 0.2, 1, and 5 Hz stimulation under normocapnia and hypercapnia. (A) Field potential recordings from the somatosensory cortex for 0.2, 1, and 5 Hz stimulation under normocapnia and hypercapnia. The recordings are representative of those of one animal. Dots indicate the points of electrical stimulation. (B) Number of field potentials detected (dotted line) and mean amplitude of potentials (solid line) under normocapnia (○) and hypercapnia (□). Note that the mean amplitude at 5 Hz was lower than those at 0.2 and 1 Hz stimuli (*p*<0.05, Student's *t*-test), though the number of field potentials increased with increasing stimulus frequency. There was no significant difference in the amplitude or number of field potentials between hypercapnia and normocapnia. Error bars indicate ±SD, *n*=9.

tion. The hind paw stimulation caused no change in the systemic arterial blood pressure during stimulation under both normocapnic and hypercapnic conditions.

Neuronal activity induced by hind paw stimulation under normocapnia and hypercapnia

In most rats under normocapnia, each electrical stimulus evoked a corresponding field potential. The mean amplitude at 5 Hz was smaller than that at 0.2 and 1 Hz stimuli (55.1±15.1% of that at 0.2 Hz, and 58.6±12.1% of that at 1 Hz; *p*<0.05) (Fig. 3). There

was no significant difference in the mean amplitude between 0.2 and 1 Hz stimulation.

The number of field potentials detected under hypercapnia was the same as that under normocapnia. On the other hand, the mean amplitudes of field potentials under hypercapnia slightly decreased compared with those under normocapnia, though a significant difference in the mean amplitude was detected in neither condition (Fig. 3B).

Effect of increasing stimulus frequency on LCBF response

Ventilation with 2.5% CO₂ for 20 min resulted in a significant increase in the baseline level of LCBF above the normocapnic levels ($p < 0.01$). The mean baseline level of LCBF under hypercapnia was about

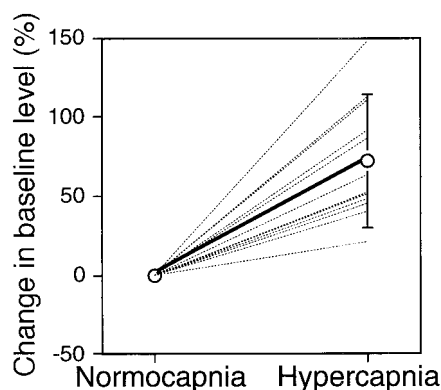


Fig. 4. Changes in baseline LCBF obtained under normocapnia and hypercapnia. Each line corresponds to data from one rat. The data were normalized to those under normocapnia after statistical analysis. Bold lines and error bars indicate the mean value and \pm SD ($n=12$), respectively. The baseline value obtained under normocapnia was considered to be zero. Note that the baseline level under hypercapnia was about 72.2% higher than under normocapnia ($p < 0.01$, Student's t -test).

72.2 \pm 42.0% higher than that under normocapnia (Fig. 4).

Under both normocapnia and hypercapnia, the absolute response magnitude of evoked LCBF in the corresponding somatosensory area after hind paw stimulation increased with increasing stimulus frequency up to 5 Hz. The absolute response magnitudes under hypercapnia at 0.2, 1, and 5 Hz stimulation were greater than those under normocapnia ($p < 0.05$), and the slope of the curve for hypercapnia is steeper than that for normocapnia ($p < 0.05$) (Fig. 5A). The differences in absolute response magnitude between normocapnia and hypercapnia at 1 and 5 Hz were, respectively, about 3.0 and 4.4 times greater than that at 0.2 Hz.

However, after normalization to each baseline level, there was no significant difference in the normalized response magnitude of the evoked LCBF between normocapnia and hypercapnia (Fig. 5B). Normalized response magnitude was also frequency dependent in the stimulation frequency range of 0.2 to 5 Hz under normocapnia and hypercapnia.

Effect of increasing stimulus frequency on time course of normalized LCBF

The time parameters of normalized LCBF response curves were calculated under different gas conditions and are listed in Table 2. The rise time of 0.5 s was nearly constant between 0.2 and 5 Hz stimulation under normocapnia and hypercapnia. Under hypercapnia, the evoked LCBF response at 1 and 5 Hz stimulations peaked later and returned to the baseline level more slowly than that under normocapnia ($p < 0.05$) (Fig. 6, Table 2). There were no statistically significant differences in the three time parameters between normo- and hypercapnia at 0.2 Hz stimulation.

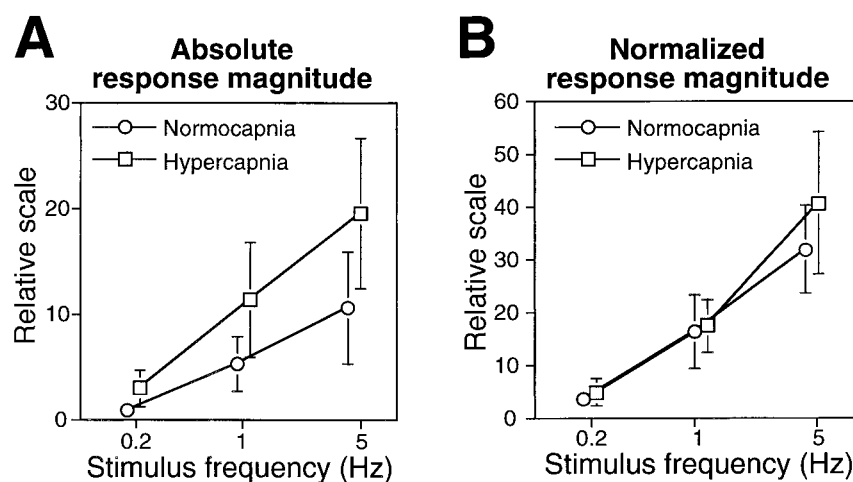


Fig. 5. Changes in response magnitude of evoked LCBF at varying frequencies under normocapnia and hypercapnia. (A) Changes in absolute response magnitude of evoked LCBF. Each value of absolute response magnitude was normalized to that at 0.2 Hz stimulation under normocapnia after statistical analysis. (B) Changes in normalized response magnitude of evoked LCBF. Note that the absolute response magnitudes under hypercapnia at 0.2, 1, and 5 Hz stimulation were greater than those under normocapnia ($p < 0.05$, Bonferroni-test), though there was no significant difference in normalized response magnitude between normocapnia and hypercapnia. Error bars indicate \pm SD, $n=12$.

Table 2. Time parameters in LCBF response curve.

Stimulus frequency	Blood gas condition	Rise time (s)	Peak time (s)	Termination time (s)
0.2 Hz	Normocapnia	0.59±0.35	2.35±0.56	4.66±1.17
	Hypercapnia	0.50±0.31	2.20±1.03	5.21±1.69
1 Hz	Normocapnia	0.56±0.20	3.50±1.21	7.49±2.06
	Hypercapnia	0.51±0.19	4.64±0.89*	9.65±1.74*
5 Hz	Normocapnia	0.54±0.27	4.20±0.55	8.18±0.97
	Hypercapnia	0.51±0.24	5.03±0.66*	12.8±1.98*

There was a significant difference in parameters between hypercapnia and normocapnia (* $p < 0.05$). Mean±SD ($n = 12$).

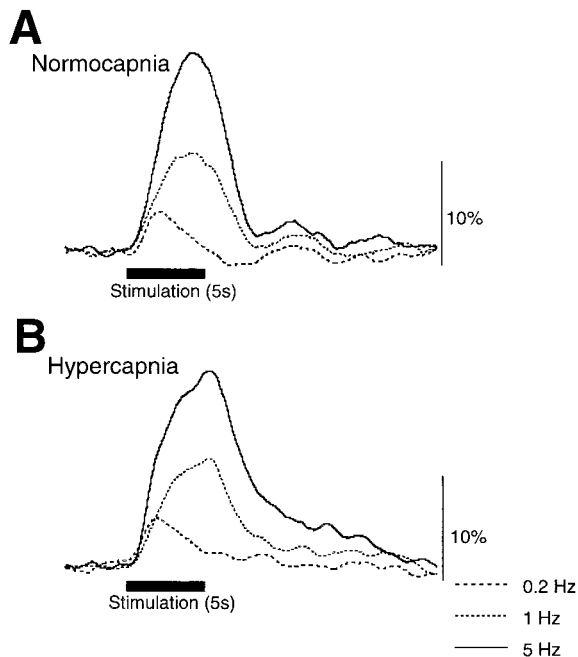


Fig. 6. Normalized response curves at varying frequencies of hind paw stimulation under normocapnia (A) and hypercapnia (B). The response curves were normalized to the baseline level and averaged by the number of animals used ($n = 12$). Note that the normalized LCBF under hypercapnia at 1 and 5 Hz stimulations peaked later and returned to the baseline level more slowly than under normocapnia ($p < 0.05$, Student's t -test), though the rise time of 0.5 s was nearly constant under both conditions regardless of the stimulus frequency.

DISCUSSION

Effect of hypercapnia on neuronal activity and evoked LCBF. Cerebrovascular response to graded somatosensory stimulation under normocapnia has been demonstrated through LCBF measurements by various techniques, including PET, fMRI, and LDF [9, 15, 16, 19, 20], though few reports evaluate the neuronal activity and that evoked LCBF [16, 21]. In the cat somatosensory cortex, the maximum hyperemia and maximum amplitude of the evoked potential were both obtained following stimulation at 2–3 Hz [21]. In the rat somatosensory cortex, it was observed

that the maximum evoked LCBF and maximum frequency of the field potential were both obtained at 5 Hz [16]. In the present study, the field potential of a localized area was measured by using an electrode inserted into the somatosensory cortex. The mean amplitude at 5 Hz was lower than those at 0.2 and 1 Hz stimulation because of inhibitory mechanisms, habituation of the peripheral nerve, or limitation in the transmission of the sensory input. On the other hand, the number of field potentials increased with the increasing stimulus frequency (Fig. 3). As a result, in our experiment the total intensity of neuronal activity during the 5 s period increased with increasing stimulus frequency up to 5 Hz. The response magnitude of the evoked LCBF also showed a frequency-dependent increase (Fig. 5). These increases suggest that the evoked LCBF reflects the intensity of neuronal activity under normocapnia.

Regardless of numerous published data about frequency-dependent changes in the evoked LCBF under normocapnia, only one report concerns the relationship between LCBF and graded stimulus frequency under hypercapnia. Gerrits *et al.* reported that the normalized LCBF in the somatosensory cortex during 13 s whisker stimulation was not statistically different between normocapnia and hypercapnia when the stimulus frequency was constant, and the initial peak, followed by a plateau under normocapnia, disappeared under hypercapnia [11]. There was no observation, however, of the effect of hypercapnia on the neuronal activity and evoked LCBF response to short-period stimulation that disrupts the biphasic response, leaving only the early transient reaction of the evoked LCBF. We investigated not only the evoked LCBF, but also neuronal activation under hypercapnia. This activity was not significantly different in comparison with that under normocapnia (Fig. 3), suggesting that in this experiment, the energy consumption was not altered by a change in the $PaCO_2$ level within the same stimulus frequency. The absolute evoked LCBF under hypercapnia increased with increasing stimulus frequency and was greater than that under normocapnia

(Fig. 5A), though the baseline level of LCBF increased under hypercapnia compared with that under normocapnia (Fig. 4). These findings suggest that the evoked LCBF is not directed toward supplying substrates for metabolism, because the substrates were supplied in abundance as a result of an increase in the baseline level of LCBF under hypercapnia. On the other hand, after normalization to each baseline level, at constant neuronal activity, the normalized LCBF under hypercapnia was not significantly different from that under normocapnia over a range of stimulus frequency of 0.2 to 5 Hz (Fig. 5B). The results of our present study confirm that the normalized evoked LCBF reflects the neuronal activity even if the stimulation period was short and suggest that the evoked LCBF is regulated by mechanisms operating on blood vessels that are proportional to the neuronal activity.

We elucidated the differences between time parameters of LCBF response curves under hypercapnia and normocapnia. Hypercapnia leads to significant increases in the peak time and termination time of LCBF response curves at 1 and 5 Hz stimulation compared with normocapnia (Fig. 6, Table 2), which were constant based on the results of a previous report [11]. This suggests that CO₂ affects the behavior of blood vessels during the return of LCBF to the baseline level. We demonstrated that the combined application of graded neuronal stimuli and a high PaCO₂ level resulted in synergistic changes in the absolute response of LCBF (see below discussion), because the difference in the absolute response magnitude at the same stimulus frequency between normocapnia and hypercapnia became large with increasing stimulus frequency (Fig. 5A)

Mechanisms underlying the combined action of graded neuronal stimuli and hypercapnia on LCBF. The mechanisms underlying the effect of hypercapnia on the relationship between graded neuronal activity and LCBF are unclear at present. At least two hypothetical mechanisms were proposed: (1) direct neurogenic control on vasculature, and (2) biochemical control, e.g., release of biochemical mediators. Hypercapnia leads to a significant decrease in MABP (Table 1). However, the effect of changes in MABP under hypercapnia on the evoked LCBF can be excluded by autoregulation of the cerebral blood flow and perfusion pressure in the brain.

It is known that there are sympathetic nerve terminals on brain vessels such as pial arterioles, and they act on smooth muscles of cerebral arteriolar walls, causing vasoconstriction and vasodilation [8, 22, 23]. There are some reports that CO₂ affects the neurogenic control of vasculature, e.g., enhancement of the

excitatory activity from the brain stem [24] and activation of chemoreceptors [25]. Moreover, the activation of neural innervation of the cerebral vessels can cause an exclusively neurogenic vasodilation because of the unchanged brain metabolism [26]. These suggest a possibility that under hypercapnia the neurons that control the vessel behavior (e.g., cholinergic and noradrenergic fibers) become more excitable, and vessels easily become dilated after excitation of the attached neurons.

Another more likely mechanism for the regulation of the flow couple is the influence of the release of biochemical LCBF-mediators under hypercapnia. LCBF is sensitive to numerous biochemical substances (K⁺, H⁺, Ca²⁺, adenosine, nitric oxide), whose activity changes during neuronal activation and under hypercapnia [2, 27–30]. It has been observed that acidification caused by CO₂ inhalation enhances nitric oxide synthase activity and K⁺ release and produces long-lasting changes in intracellular Ca²⁺ and the calmodulin activity, as well as in the adenosine release [27, 30–33]. In the present study, we demonstrated that hypercapnia caused a decline in the pH level because of a change in H⁺ concentration (Table 1). These suggest that hypercapnia may affect the enhancement of biochemical mediation of the relationship between neuronal activity and evoked LCBF through modulation of the acid–base status of blood or brain tissue, or both.

If LCBF is biochemically regulated, the production of biochemical mediators and their diffusion to cerebral vessels may be the limiting steps. Wei and Kontos calculated the diffusion time for various substances and found a very rapid mean diffusion time of <1.0 s [34]. Thus it is clear that neuronal activity and hypercapnia can influence LCBF, stimulating the release of biochemical mediators. The increase in the peak time and the termination time of the LCBF response curve under hypercapnia compared with those under normocapnia may be explained partially by the effect of CO₂ on the rate of release and the level of biochemical mediators. It may also be speculated that the exponential nature of the release of biochemical mediators changes during the graded neuronal activity. Carbon dioxide may affect this exponential function, resulting in synergism between graded neuronal activity and hypercapnia coupled to LCBF, as observed in this work. However, this hypothesis requires further verification.

The technical assistance of Mr. Y. Ito of the Akita Research Institute for Brain and Blood Vessels is gratefully acknowledged. This study was supported in part by the STA Post-

doctoral Research Fellowship from the Japan Science and Technology Agency.

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