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Doxapram stimulates the carotid body via a different mechanism than hypoxic chemotransduction

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(i) Title: Doxapram stimulates the carotid body via a different mechanism than hypoxic chemotransduction

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1. Introduction

Responses to hypoxia and hypercapnia are critical to maintaining homeostasis and involve a number of cells and organ systems. Typical responses to acute hypoxia and hypercapnia are observed in the carotid body, which monitors the partial pressure of oxygen (P_{O_2}) and partial pressure of carbon dioxide (P_{CO_2}) in arterial blood (for a review see González et al., 1994). The carotid body contains glomus (type I) cells, which produce and release neurotransmitters. The glomus cells are considered the most essential component of chemoreception since they are the only cells within the carotid body that synapse with afferent fibers (McDonald, 1981).

Generally, an influx of extracellular Ca^{2+} into the cytosol is the first step in the release of neurotransmitters from neural cells (for a review see Augustine, 2001). Likewise, in the glomus cells, an increase in intracellular Ca^{2+} is essential to signal transmission. An oxygen sensitive K^+ (K_{O_2}) channel on the membrane of the glomus cells, identified by patch clamp studies, has been implicated in initiating Ca^{2+} influx (López-Barneo et al., 1988). This hypothesis, known as "the membrane model", states that hypoxia inhibits conductance of the K_{O_2} channel, leading to depolarization of the cell membrane. Subsequently, the depolarization induces opening of voltage-dependent Ca^{2+} channels. Finally, the resultant Ca^{2+} influx releases neurotransmitters, which stimulate the terminals of afferent fibers (for a review see González et al., 1994; Lahiri et al., 2001; López-Barneo, 1996).

On the other hand, several investigators have pointed out problems with the membrane model of carotid body chemoreception (Doyle and Donnelly, 1994; Lahiri, et al., 1998; Osanai et al., 1997). The major point of argument was that inhibitors of K_{O_2}, tetraethylammonium (TEA), 4-aminopyridine (4-AP) or charybdotoxin (CTX), have no effect on the activity of sensory afferent fibers or on secretion of neurotransmitter in the whole carotid body.

Interestingly, it has been shown that doxapram, which is a respiratory stimulant (Mitchell et al., 1975; Nishino et al., 1982), inhibits the K^+ current of the glomus cells (Peers and Wyatt, 1994). These results suggest that doxapram might act through the same mechanism as hypoxia to stimulate the carotid body. The investigation of this hypothesis may provide us with a useful piece of information about the mechanism of hypoxic response in the carotid body.

The main objective of the present study is to test this hypothesis by determining if the pharmacological action of doxapram on the carotid body is the same as the physiological response to hypoxia. Therefore, we examined whether extracellular Ca^{2+} was required for the carotid body response to doxapram and whether a synergistic effect exists between hypercapnia and doxapram. These properties were observed in hypoxic chemotransduction in the carotid body (González et al., 1994; López-Barneo, 1996; Nishino et al., 1982). In addition, we studied the effects of various K^+ channel activators on carotid body chemoreception because K^+ current is thought to mediate the effects of doxapram and hypoxia on the carotid body. In the present study, we used four K^+ channel activators: pinacidil (Hamilton et al., 1989) and levcromakalim (Clapham et al, 1991) as ATP-sensitive K^+ (K_{ATP}) channel activators, NS-1619 as a large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel activator (Olsen et al., 1994), and halothane as a TASK-like background K^+ (TASK-1) channel activator (Buckler et al., 2000).

2. Methods

2.1. Animals and surgical procedures.

The Animal Experiment Committee of Asahikawa Medical College approved the present study. Twenty-one male domestic white rabbits, weighing 3.0 to 3.5 kg, were anesthetized with pentobarbital sodium (30 mg/kg, i.v.) and urethane (1200 mg/kg, i.p.). The animals were tracheostomized and ventilated mechanically (Model 665D, Harvard-Apparatus, USA). Isolated perfused carotid bodies were prepared as described in a previous paper (Osanai et al., 1996, 1997). In brief, a catheter was inserted into the common carotid artery and all other vessels were tied off except for the veins. After the carotid sinus nerve (CSN) was dissected from the glossopharyngeal nerve, the carotid artery bifurcation with the carotid body was immediately removed. The isolated carotid body was perfused with a perfusate from a constant-pressure (approximately 65 mmHg) gravity driven reservoir. The temperature of the perfusion chamber and of the fluid reservoirs was maintained by circulating warm water at 37.0 ± 0.5^{o}C. The whole CSN was immersed in a layer of liquid paraffin. The CSN was desheathed and the nerve fibers were hooked onto bipolar platinum electrodes. Fibers from the carotid sinus were cut away in order to intercept neural activity from the baroreceptor. Neural discharges were recorded using a differential amplifier (MEG-1100, Nihon-Kohden, Japan) with a notch filter (50 Hz). The impulses were monitored by an oscilloscope (VC-1100, Nihon-Kohden, Japan) and counted with an electronic amplitude discriminator and a frequency meter (MET-1100, Nihon-Kohden, Japan). Carotid chemosensory activity was recorded on a printer (RTA-1200, Nihon-Kohden, Japan) and transferred to a data acquisition system (Power Lab, AD Instrument, Australia). The resection of the fibers from the baroreceptor was confirmed by the absence of alternation of neural activity when the perfusate flow was interrupted.

2.2. Materials.

The perfusate was a modified Tyrode's solution containing 112 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.1 mM $MgCl_2$, 21.4 mM $NaHCO_3$, 5.0 mM HEPES, 5.0 mM glucose, 22.0 mM sodium glutamate, and 4 mg/mL dextran. For experiments requiring Ca^{2+}-free solution, Ca^{2+} was omitted and an equal amount of Mg^{2+} was added to the solution. The concentrations of Ca^{2+} in each solution were then measured (ICA2, Radiometer, Denmark). The normoxic and hypoxic solutions were equilibrated by bubbling with compressed gases, containing 5% CO_2 in 21% O_2, and 5% CO_2 in 3% O_2, respectively. To test the hypercapnic response, we used a normocapnic solution and two hypercapnic solutions. These were equilibrated with 5% CO_2 in 21% O_2, 7% CO_2 in 21% O_2, and 9% CO_2 in 21% O_2, respectively. In the mild hypoxic hypercapnic test, the solutions were equilibrated with 5% CO_2 in 7% O_2, 7% CO_2 in 7% O_2, and 9% CO_2 in 7% O_2, respectively. Drugs were freshly prepared prior to use. Doxapram hydrochloride (Dopram@ injectable solution, 20 mg/mL, Kissei Pharmaceutical, Japan) was diluted to the desired concentration in Tyrode's solution. Levcromakalim, pinacidil, NS-1619, and halothane were dissolved in 2 ml of dimethyl sulfoxide and stored. They were freshly diluted to adequate concentrations. The composition of gas in each solution was measured by a blood gas analyzer (ABL 300, Radiometer, Denmark), and the data are shown in Table 1.

2.3. Protocols.

(1) The CSN activity was measured by exposure to various doxapram concentrations (1 to 20 μM). (2) The effects of both hypoxia and doxapram on carotid chemoreception were tested in physiological Ca^{2+} solution and Ca^{2+}-free solution (n = 6). (3) We estimated whether doxapram had a synergistic effect on hypercapnic carotid chemoreception (n = 9). The hypercapnic test was performed using three P_{CO_2} solutions ($P_{CO_2} \approx$ 35, 45, 55 mmHg), with

and without doxapram under normoxic condition (P_{O2} ≈ 135 mmHg). Also, the hypercapnic test was performed without doxapram under mild hypoxic condition (P_{O2} ≈ 55 mmHg, P_{CO2} ≈ 35, 45, 55 mmHg). Hypercapnic chemosensitivity was evaluated in terms of the slope of CSN discharge in response to P_{CO2} (Hz/mmHg). (4) We then compared the effects of pinacidil (100 μM, n = 6), levcromakalim (50 μM, n = 6), NS-1619 (30 μM, n = 6), and halothane (100 μM, n = 6) on the carotid body response to hypoxia and doxapram. We referred to previous reports to determine the doses of K^+ channel activators (Buckler et al., 2000; Mochizuki-Oda et al., 1997; Olsen et al., 1994)

2.4. Data analysis.

Results are presented as the mean ± SEM. Statistical significance was assessed using the Student's two-tailed paired *t*-test for comparison between control and treatment groups. In order to perform a multiple comparison, analysis of variance was used. If a significant value was indicated, Dunnett's *post-hoc* tests were performed. Two-way analysis of variance was used to determine interaction between CO_2 response and effect of doxapram and interaction between CO_2 response and hypoxic response in carotid body. P was considered significant at < 0.05.

3. Results

3.1. Dose-response

The dose-response curve is shown in Fig. 1. The administration of doxapram increased the frequency of CSN impulses in a dose-dependent manner. The dose-response curve

demonstrated a maximal CSN firing response with approximately 10 μM of doxapram and so thus, this concentration was used in subsequent experiments.

3.2. The effects of doxapram on the response of the carotid body to hypercapnia

The effects of doxapram on the response of the carotid body to hypercapnia are shown in Fig. 2. The CSN activity was increased by hypercapnia (F-test: 5.14, $P < 0.01$). The CO_2 response curve was shifted to upper position by the administration of doxapram (F-test: 16.21, $P < 0.01$). However, there was no significance in interaction between the hypercapnic response and the administration of doxapram (F-test 0.04, $P = 0.96$). Also, the slope of the CO_2 response curve without doxapram was 2.7 ± 0.3 (Hz/mmHg) and that with doxapram was 3.1 ± 0.4 (Hz/mmHg). The difference between these two values was not significant. The CO_2 response curve was also shifted to upper position by hypoxia (F-test: 23.98, $P < 0.01$). In contrast, there was significance in interaction between hypercapnia and hypoxia (F-test 3.21, $P < 0.05$). Under hypoxic condition, the slope of the CO_2 response curve significantly increased to 7.2 ± 0.4 (Hz/mmHg) ($P < 0.05$). Thus, doxapram stimulated the carotid body and increased CSN discharge, but a synergistic interaction between hypercapnia and doxapram on carotid body chemoreception was not observed. Meanwhile, hypoxia interacted to hypercapnic carotid chemosensitivity.

3.3. The effect of hypoxia and doxapram on CSN activity in Ca^{2+}-free solution

The effect of hypoxia and doxapram on CSN activity in Ca^{2+}-free solution is illustrated in Fig. 3. After switching to a Ca^{2+}-free solution, the CSN response to hypoxia decreased. Also, the response to doxapram diminished during perfusion of the Ca^{2+}-free solution. The effects of Ca^{2+}-free solution on carotid body responses to hypoxic and doxapram are summarized in Fig.

4. The Ca^{2+}-free solution decreased carotid body chemoreceptor responses to hypoxia and doxapram.

3.4. Effects of K^+ channel activators in carotid sinus response to hypoxia and doxapram

The effects of K^+ channel activators on responses of the carotid body to hypoxia and doxapram are summarized in Table 2. The administration of pinacidil altered neither the hypoxic response nor the response to doxapram in the carotid body. Also, there was little effect of levcromakalim on CSN responses to hypoxia and doxapram.

The effects of NS-1619 on responses of the carotid body to hypoxia and doxapram are shown in Table 2 and Fig. 5. The administration of NS-1619 did not change the CSN activity during normoxia and the CSN response to hypoxia. However, the CSN response to doxapram was partially decreased by co-administration of NS-1619. After washout of NS-1619, the CSN discharge was not altered during normoxic and hypoxic condition. Also, the CSN response to doxapram was recovered by the washout of NS-1619. Therefore, it is unlikely that the inhibitory effect of NS-1619 on the CSN response to doxapram was nonspecific effect.

A real record of CSN activity in response to hypoxia and doxapram with halothane is shown in Fig. 5. The administration of halothane decreased CSN activity under normoxic conditions. In addition, the CSN responses to hypoxia and doxapram were significantly blocked by halothane (Table 2).

4. Discussion

Previous studies using *in vivo* carotid bodies have shown that the stimulus effect of doxapram is independent of arterial P_{O_2} or P_{CO_2} (Mitchell et al., 1975; Nishino et al., 1982). The results obtained in the present study are consistent with these reports because a

synergistic effect between doxapram and hypercapnia was not observed. This is different from the physiological effects of hypoxia on the carotid body chemotransduction.

The combined effect of hypoxia and hypercapnia on carotid body chemotransduction remains unclear. Recent reports demonstrate an interaction between their combined effects on intracellular Ca^{2+} concentrations in glomus cells (Dasso et al., 2000; Roy et al., 2000). However, Roy et al. (2000) noted a discrepancy in terms of the CSN response to combined hypoxia and hypercapnia and the intracellular Ca^{2+} response within glomus cells. Therefore, it seems that CSN firing is regulated by additional factors.

It is recognized that Ca^{2+} is an indispensable factor in carotid body chemoreception because the response of glomus cells to chemical stimulation is abolished by Ca^{2+} channel blockers or removal of extracellular Ca^{2+} (López-Barneo., 1996). Moreover, intracellular Ca^{2+} is increased in glomus cells of the carotid body at the time of neurotransmitter release (Delpiano et al., 1989). Since a previous study showed that doxapram releases the neurotransmitter from isolated glomus cells and doxapram-evoked release is inhibited by the Ca^{2+} channel blocker (Anderson-Beck et al., 1995), the influx of Ca^{2+} must be necessary for the effect of doxapram on the carotid body chemotransduction.

It has been postulated that a heme-like protein plays an important role in the carotid body response to hypoxia and hypercapnia (Lahiri et al., 1975; Osanai et al., 1996). The oxygen binding capacity of heme is inhibited by various metabolic inhibitors, including cyanide and carbon monoxide. This characteristic of the heme group might explain why metabolic inhibitors synergistically augment the hypercapnic carotid body response, similar to that observed when hypercapnia is combined with hypoxia. In addition, a number of allosteric effecters (i.e. H^{+}) can alter hemes affinity for O_2. Therefore, if a heme-like protein serves as an oxygen sensor in the carotid body, the detection of oxygen might be altered by changes in the affinity of heme for oxygen, such as might occur with alterations in pH. A heme-like oxygen

sensor might mediate the combined effect of O_2 and CO_2 on the carotid body. It has also been shown that almitrine, which is another respiratory stimulant, partially augments the response of the carotid body to hypercapnia (Lahiri et al., 1989). Recently, an inhibitory effect of almitrine on metabolism has been reported (Leverve et al., 1994). In contrast, doxapram has no inhibitory effect on metabolism (Cote et al., 1992). The different pathways by which these stimulate carotid body chemotransduction might also be responsible for the differences in their effects on metabolism.

Recently, hypoxia has been observed to inhibit several types of K^+ channels, each of which produces a characteristic K^+ current in glomus cells. The following four types of K^+ currents have been identified and investigated in relation to their response to hypoxia in glomus cells: (1) Ca^{2+}-insensitive, voltage-dependent transient K^+ current (López-Barneo et al., 1988); (2) Ca^{2+}-sensitive, voltage-dependent K^+ current, which is similar to BK_{Ca} (Peers, 1991; Wyatt et al., 1994); (3) TASK-like background K^+ current (Buckler et al., 2000); (4) HERG-like K^+ current (Overholt et al., 2000). In light of the results of previous investigations, we hypothesized that K^+ channel activators might have an effect on carotid chemoreceptor activation in response to hypoxia.

The first study to investigate the effect of ATP on KO_2 channels in glomus cells found that the K^+ current was not altered by application of ATP to the inside of the cytosol membrane (López-Barneo et al., 1988). Since this report, no other studies have investigated the effect of hypoxia on K_{ATP} current in glomus cells. In the present study, the effects of two types of K_{ATP} channel activators were investigated. The results suggest that K_{ATP} channels have no physiological effect in carotid body chemotransduction. It is possible that only a small density of K_{ATP} channels exists on glomus cells. Electrophysiological evidence of KCa, KO_2 and small conductance K^+ channels on glomus cells is restricted to glomus cells of the rabbit

and there has been little description of K_{ATP} current on glomus cells (Ganfornina et al., 1992). Based on these findings, it is possible that K_{ATP} channels exist at very low density.

Doxapram has been reported to inhibit both Ca^{2+}-insensitive and Ca^{2+}-sensitive K^+ channels on glomus cells. The Ca^{2+}-sensitive K^+ channels were sensitive to voltage and inhibited by CTX, which is a selective BK_{Ca} channel blocker (Peers, 1991; Wyatt et al., 1994). The present study showed partial inhibition of the effects of doxapram on the carotid body with NS-1619, which supports the results of previous reports. However, the response of the carotid body to hypoxia was not influenced by NS-1619 in the present study. This provides further evidence that the effects of doxapram differ from those of hypoxia with regard to carotid body chemotransduction.

On the other hand, nonspecific K^+ channel blockers, including TEA, 4-AP, and CTX, have been observed to block K_{O2} current in patch-clamped glomus cells (López-Barneo, 1988). These K^+ channel blockers, however, did not elicit CSN activity (Doyle et al., 1994; Lahiri et al., 1998; Osanai et al., 1997). A recent report has shown that hypoxia and K^+ channel blockers (TEA and iberiotoxin) induce catecholamine secretion in thin slices of carotid body, using patch-clamp and amperometry recordings. The authors discussed the reasons why K^+ channel blockers were not observed to stimulate CSN discharge in whole carotid body preparations. They suggested that the blockers might not have diffused into the extracellular space adjacent to cells of the carotid body and thus could not inhibit K^+ current. The present study, however showed inhibition of the effects of doxapram on the perfused carotid body by NS-1619; therefore, NS-1619 must have reached the glomus cells. If the BK_{Ca} channel is the primary O_2 sensor in the glomus cells, there might be some chemotransductive response to hypoxia. These findings suggest that doxapram may exert its effect on the carotid body via inhibition of BK_{Ca} current; however, inhibition of BK_{Ca} current cannot be involved in the initial response of the carotid body to hypoxia.

Buckler et al. (2000) have reported expression of oxygen- and acid-sensitive background K^+ channels, which are likely to be TASK1-like channels, in glomus cells. Recently, it has been published that the biophysical properties of these channels are identical with either TASK-1 or TASK-3 (Williams et al., 2004). The activity of these channels is related to cytosolic concentration of ATP and inhibition of oxidative phosphorylation. Therefore, TASK-like background K^+ channels on glomus cells may detect hypoxia via alternation of cellular energy metabolism.

It has also been shown that halothane stimulates TASK-like background K^+ channels on glomus cells. Therefore, carotid body chemosensitivity to hypoxia may be inhibited by administration of halothane. It is noteworthy that general anesthetics, including halothane, enflurane and isoflurane, have been found to reduce the hypoxic ventilatory response (Hirshman et al., 1977). In the present study, the effect of halothane on carotid body chemosensory responses to hypoxia is consistent with the observations of previous reports. Also, TASK-like K^+ current within the carotid body is inhibited by acidosis (Buckler et al., 2000). This finding might correspond to data indicating that halothane reduces CSN activity due to hypercapnia (Davies et al., 1982; Nishino et al., 1982). Also, halothane has been observed to reduce carotid body excitation by nicotine (Cote et al., 1992). Therefore, it is possible that halothane affects some critical mechanism of chemotransduction in the glomus cells.

We conclude that doxapram might increase chemosensory discharge via inhibition of BK_{Ca} current within the carotid body. To the best of our knowledge, this is the first report about pharmacological antagonist of doxapram in the carotid body. Since the CSN response to hypoxia was not influenced by the inhibition of BK_{Ca} and doxapram did not interact to the CSN response to hypercapnia, the effect of doxapram may differ from that which governs hypoxic chemotransduction. Those results were obtained from *ex vivo* experiments and there

was limitation to evaluate intracellular mechanisms of the carotid body chemotransduction. It is necessary for further elucidation to measure alternation of intracellular Ca^{2+} or neurotransmitters release by administration of doxapram and its inhibitor.

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Figure legends

Figure 1. Dose-response curve for the effect of doxapram on carotid body chemosensory discharge. The data represent the mean ± SEM (n = 6). * $P < 0.05$ compared to control.

Figure 2. The carotid chemosensory response to three levels of P_{CO2}, without doxapram (open circles), with doxapram (open squares) and under hypoxia (closed triangles). The data represent the mean ± SEM (n = 9).

Figure 3. A real record of carotid sinus nerve (CSN) discharge in response to hypoxia and doxapram in an isolated-perfused rabbit carotid body. The CSN discharge is plotted against time.

Figure 4. Carotid sinus nerve (CSN) discharge in response to hypoxia and normoxia with doxapram, using Ca^{2+}-free perfusate. Histograms illustrate increases of CSN discharge from control (ΔCSN) in normal Ca^{2+} solution (open bars) and Ca^{2+}-free solution (shaded bars). The concentration of Ca^{2+} in each solution is shown below each bar.

Figure 5. Effects of NS-1619 on carotid sinus nerve (CSN) discharge in response to normoxia, hypoxia, and normoxia with doxapram. Histograms illustrate CSN discharge without NS-1619 (open bars), with NS-1619 (closed bars) and after washout of NS-1619 (shaded bars). The data represent the mean ± SEM (n = 6).

Figure 6. A real record of carotid sinus nerve (CSN) discharge in response to hypoxia and doxapram in an isolated-perfused rabbit carotid body. The CSN discharge is plotted against time.

Table 1

	pH	P_{CO_2} (mmHg)	P_{O_2} (mmHg)
control	7.41 ± 0.02	34.6 ± 0.9	114.5 ± 3.9
mild hypercapnia	7.29 ± 0.01*	44.4 ± 0.7*	123.2 ± 5.1
hypercapnia	7.21 ± 0.01*	55.0 ± 1.1*	113.5 ± 3.6
normocapnic hypoxia	7.39 ± 0.02	36.4 ± 0.4	56.9 ± 3.2*
mild hypercapnic hypoxia	7.28 ± 0.01*	44.4 ± 0.7*	54.8 ± 2.7*
hypercapnic hypoxia	7.20 ± 0.01*	53.0 ± 1.1*	52.8 ± 3.5*
hypoxia	7.43 ± 0.01	33.4 ± 0.3	24.8 ± 2.2*

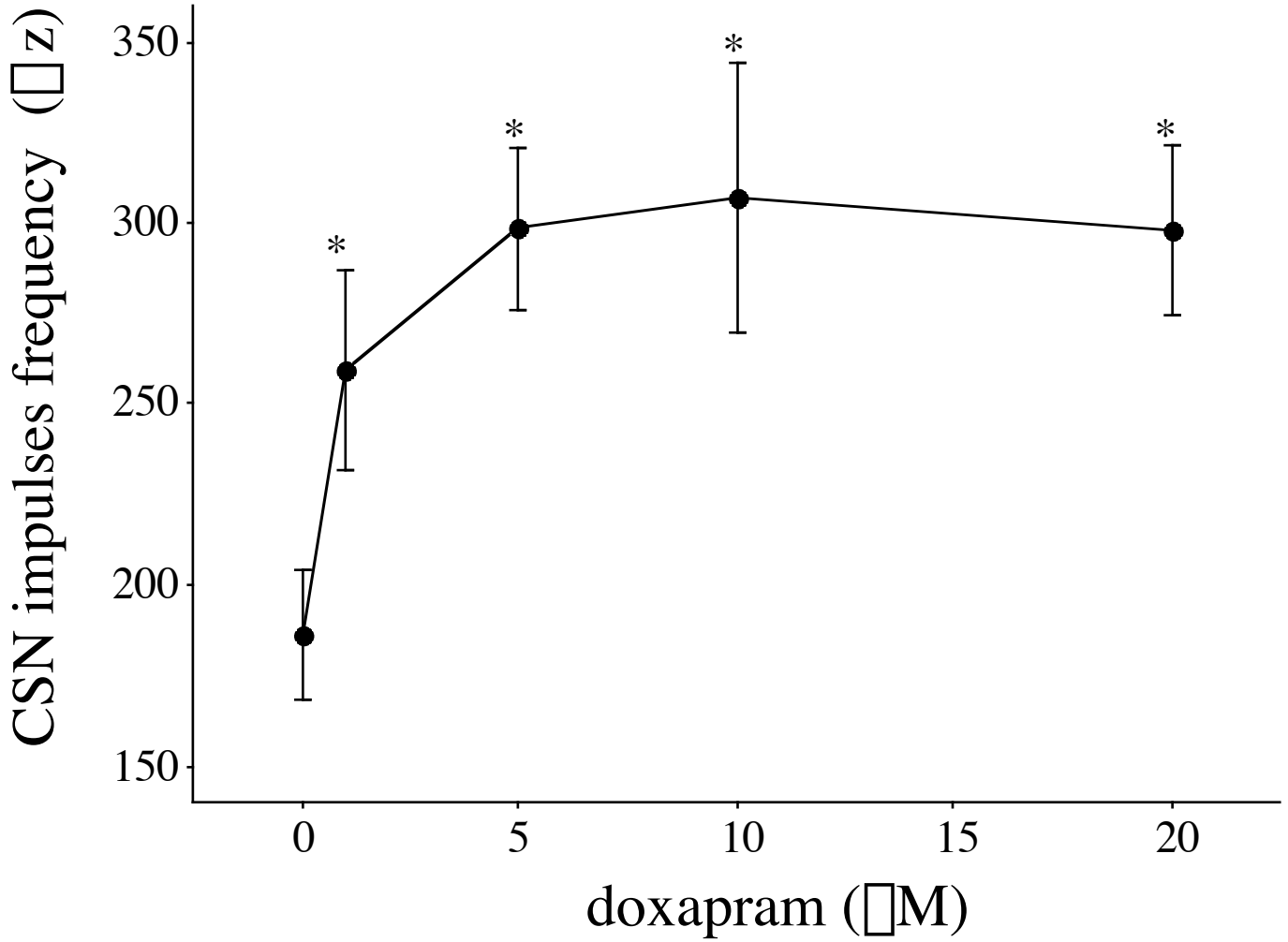
* $P < 0.05$ compared to control solution.

Table 2

	ΔCSN impulses frequency (Hz)
hypoxia	153.1 ± 32.7
hypoxia + pinacidil	141.1 ± 37.2
doxapram	59.1 ± 11.7
doxapram + pinacidil	67.3 ± 23.4
hypoxia	178.0 ± 54.6
hypoxia + levocromakalim	153.1 ± 51.6
doxapram	57.6 ± 11.4
doxapram + levocromakalim	45.8 ± 12.9
hypoxia	123.0 ± 19.2
hypoxia + NS1619	115.7 ± 17.9
doxapram	35.3 ± 8.8
doxapram + NS1619	10.2 ± 7.6*
hypoxia	123.8 ± 25.8
hypoxia + halothane	109.5 ± 16.5*
doxapram	27.2 ± 10.1
doxapram + halothane	14.3 ± 8.6*

*$P < 0.05$, hypoxia vs. hypoxia with K^+ channel opener or doxapram vs. doxapram with K^+ channel opener. ΔCSN: increase of carotid sinus nerve discharge from control.

Fig. 1


CSN impulses frequency (□z)
350
300
250
200
150
0
5
10
15
20
doxapram (□M)
*
*
*
*

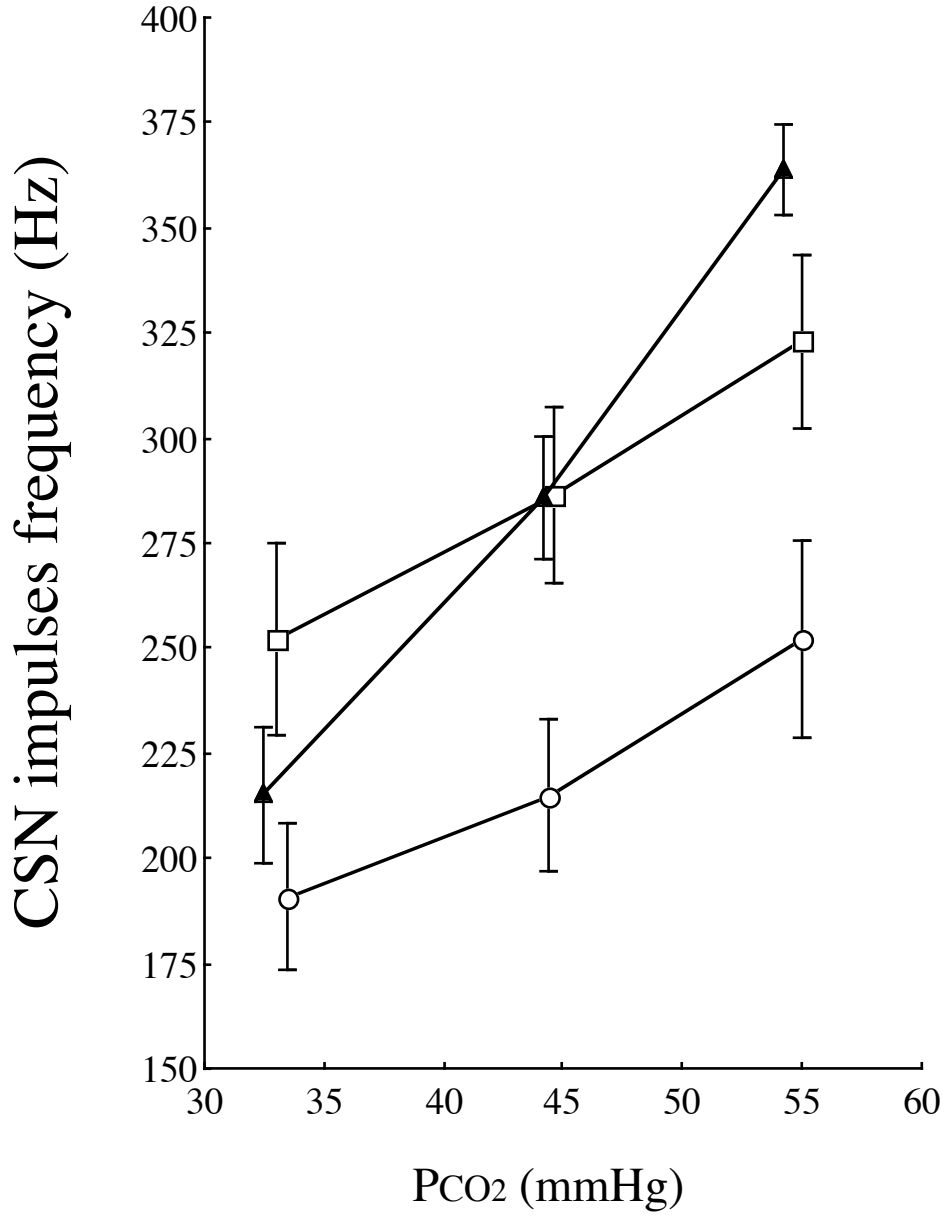

CSN impulses frequency (Hz)
400
375
350
325
300
275
250
225
200
175
150
30
35
40
45
50
55
60
PCO2 (mmHg)

Fig. 3

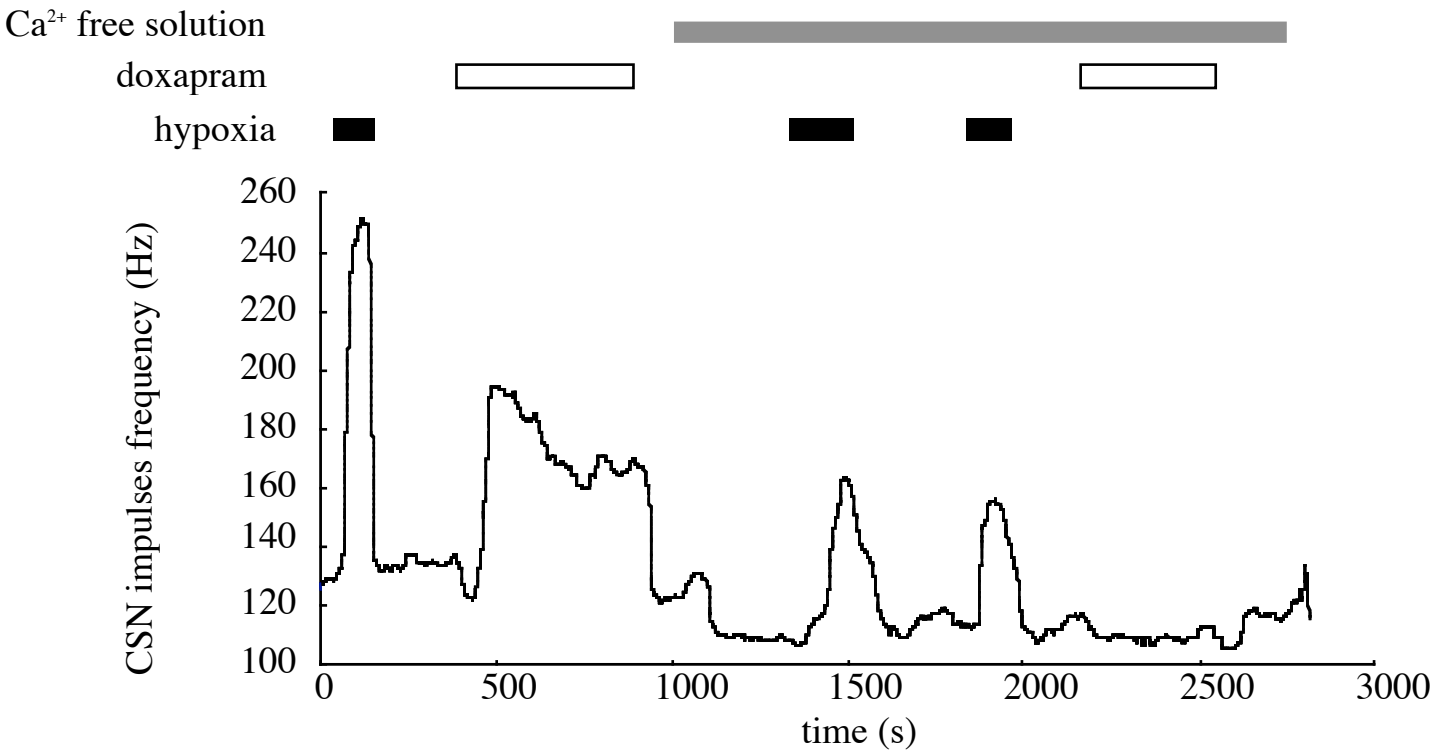
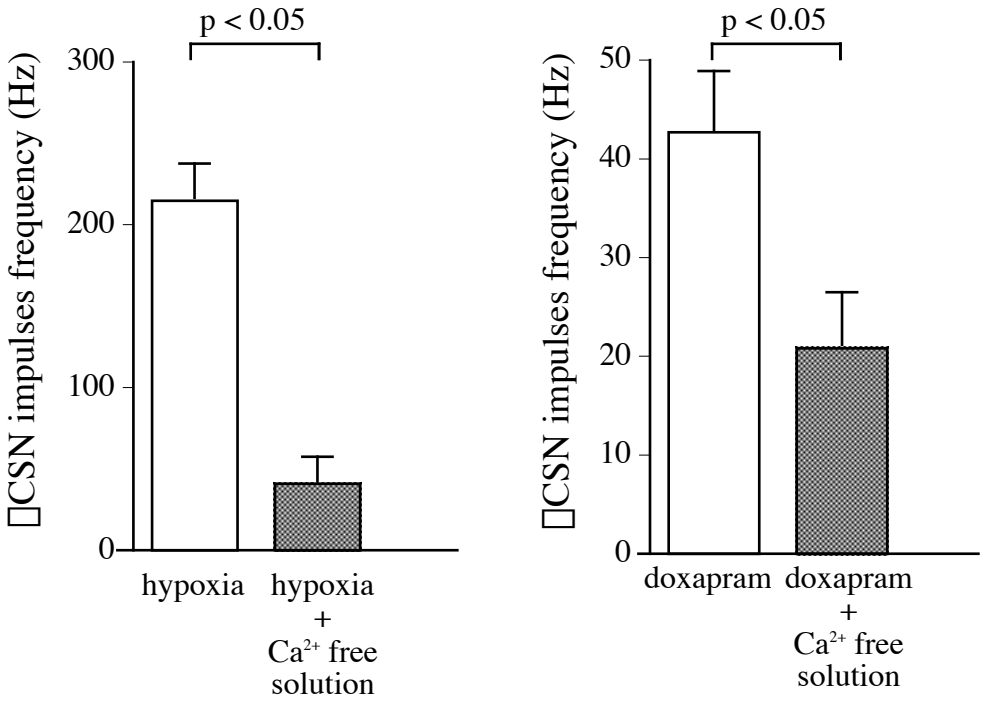
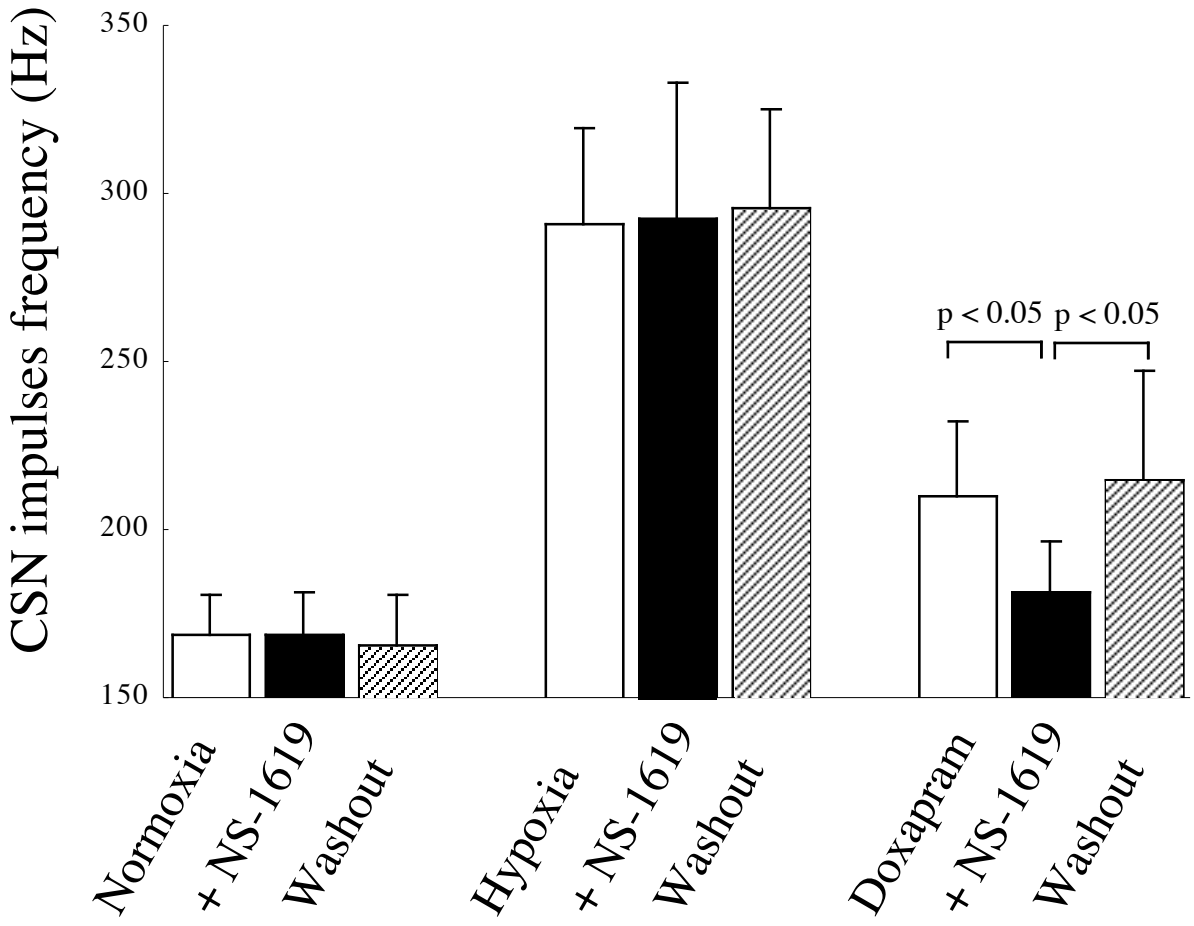

Ca2+ free solution
doxapram
hypoxia
CSN impulses frequency (Hz)
260
240
220
200
180
160
140
120
100
0
500
1000
1500
2000
2500
3000
time (s)

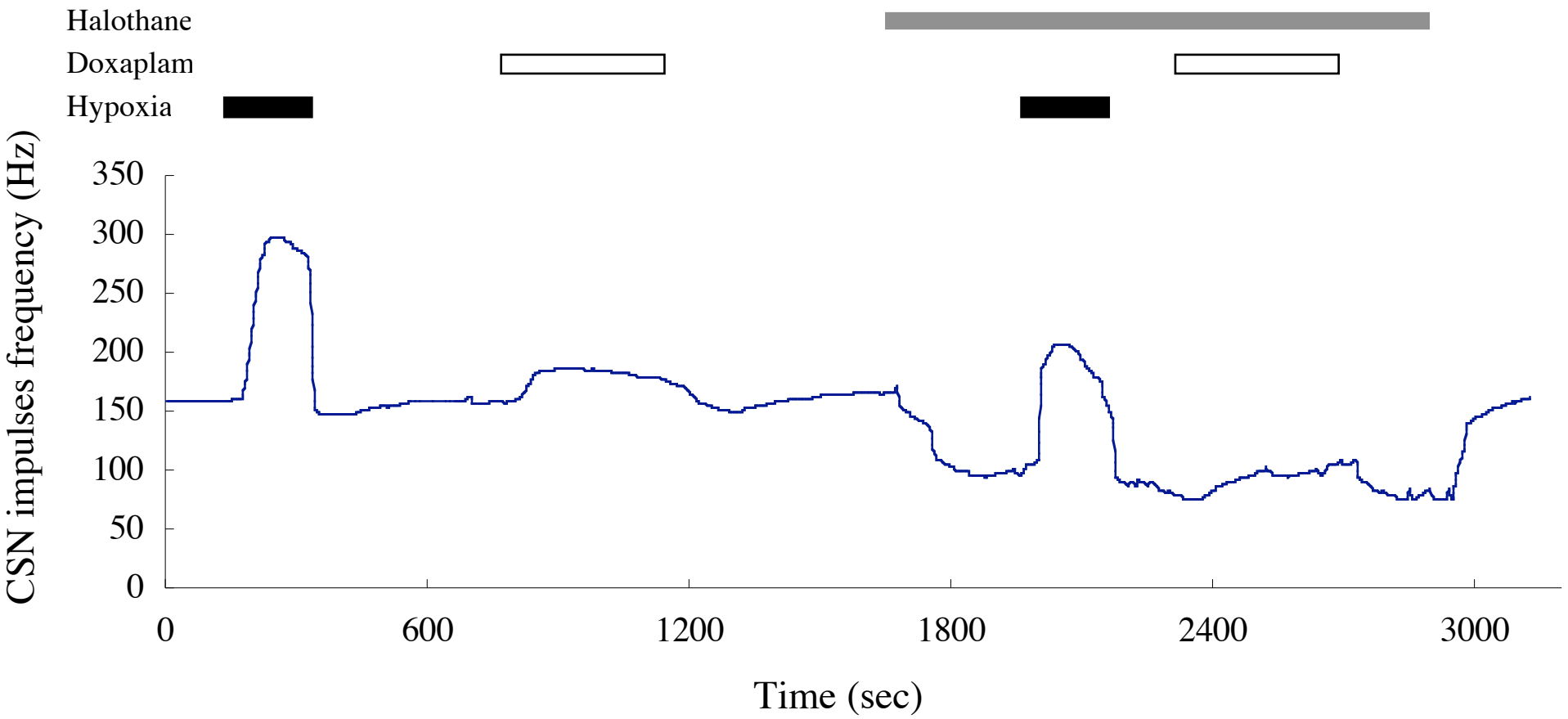
Fig. 4



[Ca^{2+}] in solution (mmol/L)	1.70 ± 0.02	0.33 ± 0.01	1.66 ± 0.03	0.32 ± 0.01

Fig. 5


CSN impulses frequency (Hz)
350
300
250
200
150
p < 0.05
p < 0.05
Normoxia
+ NS-1619
Washout
Hypoxia
+ NS-1619
Washout
Doxapram
+ NS-1619
Washout


Halothane
Doxaplam
Hypoxia
CSN impulses frequency (Hz)
350
300
250
200
150
100
50
0
0
600
1200
1800
2400
3000
Time (sec)