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Aerobic metabolism on muscle contraction in porcine gastric smooth muscle

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ABSTRACT. Exposure to chronic hypoxic conditions causes various gastric diseases, including gastric ulcers. It has been suggested that gastric smooth muscle contraction is associated with aerobic metabolism. However, there are no reports on the association between gastric smooth muscle contraction and aerobic metabolism, and we have investigated this association in the present study. High K^+- and carbachol (CCh)-induced muscle contractions involved increasing O_2 consumption. Aeration with N_2 (hypoxia) and NaCN significantly decreased high K^+- and CCh-induced muscle contraction and O_2 consumption. In addition, hypoxia and NaCN significantly decreased creatine phosphate (PCr) contents in the presence of high K^+. Moreover, decrease in CCh-induced contraction and O_2 consumption was greater than that of high K^+. Our results suggest that hypoxia and NaCN inhibit high K^+- and CCh-induced contractions in gastric fundus smooth muscles by decreasing O_2 consumption and intracellular PCr content. However, the inhibition of CCh-induced muscle contraction was greater than that of high K^+-induced muscle contraction.

KEY WORDS: gastro intestine, hypoxia, smooth muscle

Hypoxia reduces the contraction of skeletal and cardiac muscles [11, 14]; similarly, the contraction of smooth muscles is also reduced in hypoxia. However, changes in the contraction of smooth muscle differ between organs and tissues. Aeration of N_2 instead of O_2, abolishes high K^+-induced contraction in the guinea pig taenia coli [3, 4, 20, 21] and porcine iris sphincter muscle [8]. In addition, sodium cyanide (NaCN), aerobic metabolism inhibitor, significantly suppresses the high K^+-induced contraction in guinea pig urinary bladder [6]. In contrast, some researchers have shown that hypoxia has minor effects on the contraction of arterial smooth muscles, like that of the aorta and carotid artery [13]. Moreover, hypoxia mildly reduces high K^+-induced contraction of the smooth muscle of bovine trachea [7].

Exposure to chronic hypoxic conditions causes various gastric diseases, such as gastric ulcers [2, 19]. Recent study showed that hypoxic conditions in gastric cancer lead overexpression of cellular prion proteins [12], which confers multidrug resistance, promoting metastasis and inhibiting apoptosis in gastric cancer. Conversely, effects of exposure to acute hypoxic conditions, such as mountain sickness can cause anorexia, headache, vertigo, nausea, and vomiting. Yoshimoto, *et al*. [23] have reported that exposure to 0.5 atmosphere absolute, which is equivalent to a height of 5,065 m, reduced the area of gastric contraction waves in rats. These reports suggest that oxygen supply to the gastric smooth muscle is a significant factor of gastric motility. However, there are a few reports about the effects of super acute hypoxia on gastric physiological function and oxygen requirement. Because, it is very difficult to obtain human stomach tissue, porcine tissue has been used as a large-animal model to study the physiology and pathophysiology of the stomach as its function are similar to those of the human stomach [1].

In the present study, we measured muscle contraction, oxygen consumption, creatine phosphate (PCr) and adenosine triphosphate (ATP) to examine the effects of hypoxia on high K^+- and carbachol (CCh)-induced contractions of gastric longitudinal or circular smooth muscle.

MATERIALS AND METHODS

Muscle preparations and tension measurement

The stomachs from adult pigs of either sex were obtained from a local abattoir. The mucosal layer was removed by cutting with fine scissors, and strips of circular and longitudinal smooth muscle were isolated from the fundus region. Muscle strips

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(approximately 2 mm in width and 7–8 mm in length) were incubated in physiological salt solution (PSS) containing (in mM) 136.8 NaCl, 5.4 KCl, 11.9 $NaHCO_3$, and 5.6 glucose. PSS was aerated with 95% O_2 and 5% CO_2 to adjust the pH to 7.2 at 37°C. In hypoxic conditions, PSS was aerated with 95% N_2 and 5% CO_2 instead of 95% O_2 and 5% CO_2, and pH did not change even in hypoxic condition.

Muscle tension was recorded isometrically. One end of each strip was bound to a glass holder and the other end was connected by a silk thread to a strain-gauge transducer (TB-611T; Nihon Kohden, Tokyo, Japan) in an organ bath containing PSS with a resting tension of 2.0 g. Muscle strips were equilibrated for 30 min to obtain stable contractility induced by hyperosmotic 65 mM KCl (H-65K^+). The developed tension was expressed as a percentage by assuming the values at rest in PSS to be 0% and those at 15 min after addition of H-65K^+ or 0.3 μM CCh to be 100%. In the present study, we tentatively expressed decrease in muscle contraction as relaxation.

Oxygen consumption measurement

Two types of muscle strips cut from gastric fundus, circular and longitudinal smooth muscles, were incubated with PSS for 30 min. After incubation, the muscles were placed into an organ bath with PSS (3 m*l*) saturated with oxygen at 37°C. In addition, we measured oxygen consumption under each condition (control, added H-65K^+ or 0.3 μM CCh and NaCN). O_2 consumption rates were obtained 10 min after the application of each reagent. Each condition was measured for 15 min by a Clark-type polarograph electrode (YSI) connected with a biological oxygen monitor (YSI model 5300; YSI Japan Corp., Tokyo, Japan).

Assay of creatine phosphate and adenosine triphosphate

Creatine phosphate (PCr) and adenosine triphosphate (ATP) content of the muscle strips was measured using high-performance liquid chromatography (HPLC). Muscle strips were incubated with PSS for 30 min and contracted by H-65K^+ or 0.3 μM CCh. After contraction, NaCN was added or the muscle strips were aerated with 95% N_2 and 5% CO_2 for 20 min. The muscle strips were then rapidly frozen in liquid nitrogen and stored at −80°C until homogenization in 9% perchloric acid (0.3 m*l*). The homogenate was centrifuged at 15,000 × g for 5 min, and the supernatant was neutralized using 0.25 m*l* of 2 M $KHCO_3$. The neutralized extracts were centrifuged again and 20 μl supernatant was applied to the HPLC.

The HPLC system (Shimadzu Corp., Kyoto, Japan) consisted of a pump (LC-10AT), a system controller (SCL-10AT), an auto injector (SIL-10AF), a column oven (CTO-10A), and wave length-selectable detector (SPD-10Ai) set at 216 nm.

Chromatography was performed by μRPC C2/C18 ST (4.6 mm internal diameter and 100 mm length, Amersham Biosciences, Piscataway, NJ, U.S.A.) using mobile phases of 50 mM KH_2PO_4 and 5 mM terabutylammoninum hydrogen sulfate (TBAHS) (pH 6.0, buffer A), and 50 mM TBAHS and 40% methanol (pH 6.0, buffer B). Flow rate was 1.0 m*l*/min and the elution started with 65% buffer A. In the first 14 min, the flow of buffer B increased at a rate of 2.5%/min. This was followed by elution with 70% buffer B for 20 min and then with 100% buffer A for 10 min. These procedures were programmed using a system controller. The sensitivity of the detector was usually set at 1.0 AUFS and the oven temperature at 40°C. PCr and ATP contents were expressed as μmol/g wet weight.

Chemicals

CCh (Sigma-Aldrich, St. Louis, MI, U.S.A.) and NaCN (Wako Pure Chemical, Osaka, Japan) were used.

Statistics

Values are expressed as mean ± S.E.M. Statistical analyses were performed by Student's *t*-test and two-way analysis of variance, followed by the Bonferroni *post-hoc* test. Calculations and statistical analyses were performed using GraphPad Prism7 and Excel 2010 for Windows. $P<0.05$ or $P<0.01$ was considered significant.

RESULTS

Effects of NaCN and hypoxia on high K^+- and CCh-induced tension in the porcine gastric smooth muscle

Strips of longitudinal or circular smooth muscle were contracted using H-65K^+ or 0.3 μM CCh. H-65K^+ induced a tonic contraction in the longitudinal and circular smooth muscle of the porcine fundus (Fig. 1A and 1C). On the other hand, 0.3 μM CCh also induced a large phasic contraction followed by a tonic one in the longitudinal and circular smooth muscles (Fig. 1B and 1D). The effects of aeration with N_2 instead of O_2 on H-65K^+- (Fig. 2B) and CCh-induced contraction were also investigated (Fig. 2C). As shown in Fig. 2, hypoxia reduced H-65K^+- and CCh-induced contraction to 49.3 ± 8.2% and 8.9 ± 3.4% in longitudinal smooth muscle, respectively. Moreover, NaCN inhibited H-65K^+- and CCh-induced contraction in a concentration-dependent manner in longitudinal and circular smooth muscles (Fig. 1E). However, the relaxations by hypoxia and NaCN were greater in CCh-induced contraction than in H-65K^+-induced contraction (Figs. 1E, 2C and 2D). Moreover, the NaCN-induced inhibition of CCh-enhanced contraction was stronger in longitudinal muscle than in circular muscle (Fig. 1E).

Effects of NaCN on high K^+- and CCh-induced increases in O_2 consumption

In longitudinal or circular smooth muscles, the rate of basic O_2 consumption measured for 10 min was 0.016 ± 0.006 or 0.017 ± 0.004 μM/g /min, respectively. In the application of H-65K^+ to longitudinal or circular smooth muscle, the rate of O_2 consumption increased to 0.024 ± 0.007 or 0.025 ± 0.005 μM/g /min, respectively. The increased rate of O_2 consumption was significantly reduced by the addition of NaCN (1 mM). However, 300 μM NaCN did not have an effect on H-65K^+-induced increases in O_2

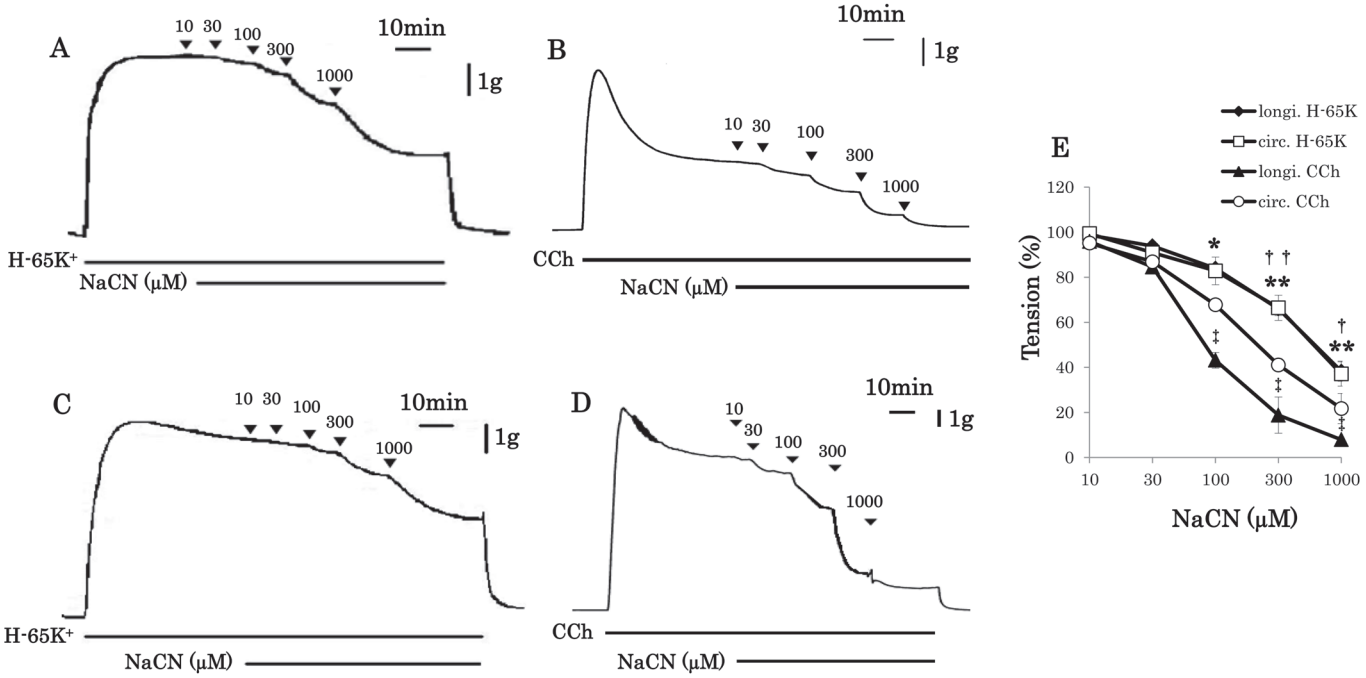

Fig. 1. Effects of applied NaCN (10 μM–1 mM) cumulatively on the H-65K$^+$- and CCh-induced contraction in longitudinal and circular smooth muscle. A typical trace of longitudinal (A, B) and circular (C, D) smooth muscle contraction. E: Concentration–response association for NaCN-induced reduction obtained at the sustained phase of H-65K$^+$ or CCh contraction. Each point represents the mean of 4 preparations. Vertical bars indicate S.E.M. * and ** indicate significant differences between H-65K$^+$ and CCh in longitudinal muscle P<0.05 or at P<0.01 (n=4), respectively. † and †† indicate significant differences between H-65K$^+$ and CCh in circular muscle at P<0.05 or P<0.01 (n=4–5), respectively. ‡ indicate significant differences between longitudinal and circular smooth muscle contraction induced by CCh (0.3 μM) at P<0.01 (n=4–5), respectively.

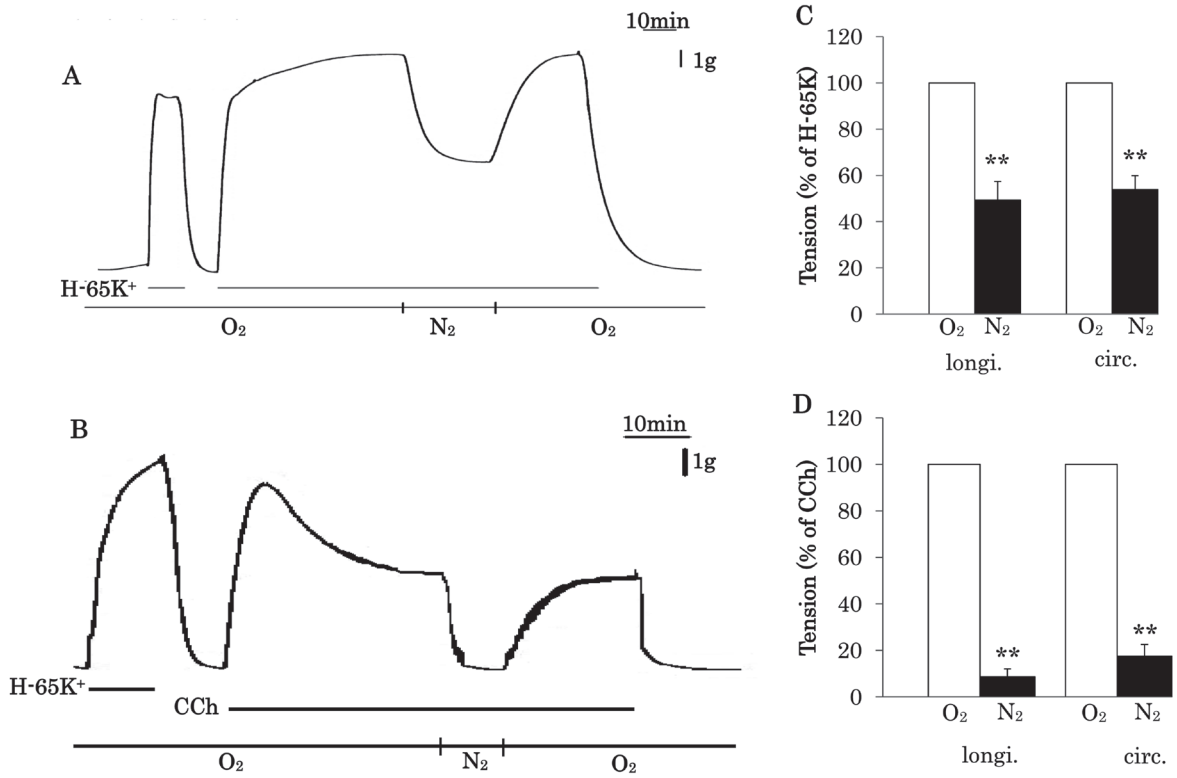

Fig. 2. Effects of hypoxia (aerated 95% N_2, 5% CO_2) on the H-65K$^+$- or CCh-induced contraction in longitudinal and circular smooth muscle. Typical trace of hypoxia-induced inhibition of H-65K$^+$ (A)- or CCh (B)-induced contraction in longitudinal muscle. Values of aeration with N_2 instead of O_2 in presence of H-65K$^+$ (C) and 0.3 μM CCh (D) were obtained 10 min after changing the aeration from O_2 to N_2. Values at 10 min were assigned as 100%. The data are expressed as means ± S.E.M. (n=4). ** Significantly different from the O_2 column (P<0.01).

Table 1. Changes in tension and rate of O_2 consumption of longitudinal and circular smooth muscle in various conditions

Condition	Longi.		Circ.	
	Tension (%)	O_2 consumption (μM/g/min)	Tension (%)	O_2 consumption (μM/g/min)
Resting		0.016 ± 0.006		0.017 ± 0.004
H-65K$^+$	100	0.024 ± 0.007 [a)]	100	0.025 ± 0.005 [a)]
+NaCN (300 μM)	66.0 ± 4.5	0.027 ± 0.012	66.4 ± 5.4	0.025 ± 0.004
+NaCN (1 mM)	38.3 ± 3.2	−0.003 ± 0.006 [b)]	37.1 ± 5.6	−0.001 ± 0.005 [c)]
CCh (0.3 μM)	100	0.019 ± 0.003	100	0.019 ± 0.004
+NaCN (300 μM)	18.8 ± 1.3	0.010 ± 0.003 [b)]	41.1 ± 6.7	0.010 ± 0.002 [b)]
+NaCN (1 mM)	7.9 ± 1.6	0.000 ± 0.001 [c)]	21.7 ± 4.6	0.009 ± 0.005 [c)]

The rate of O_2 consumption was measured 10 min after the addition of NaCN (300 μM,1 mM). The data are expressed as means ± S.E.M. (n=4) a) P<0.01 (v.s. resting), b) P<0.05, c) P<0.01 (v.s. H-65K$^+$).

Table 2. PCr and ATP contents of longitudinal and circular smooth muscle in various conditions

Condition	PCr contents (μM/g wet wt)		ATP contents (μM/g wet wt)	
	Longi.	Circ.	Longi.	Circ.
H-65K$^+$	0.76 ± 0.20 (3)	0.52 ± 0.10 (5)	0.18 ± 0.07 (3)	0.29 ± 0.09 (5)
+NaCN (300 μM)	0.60 ± 0.09 (4)	0.45 ± 0.06 (5)	0.16 ± 0.02 (4)	0.23 ± 0.10 (5)
+NaCN (1 mM)	0.39 ± 0.09 (5) [a)]	0.39 ± 0.05 (5)	0.26 ± 0.09 (5)	0.25 ± 0.07 (5)
+N_2	0.32 ± 0.07 (5) [a)]	0.28 ± 0.04 (4) [a)]	0.16 ± 0.05 (5)	0.25 ± 0.09 (4)

PCr and ATP contents were measured 20 min after addition of H-65K$^+$, NaCN (300 μM,1 mM) or aeration 95% N_2, 5% O_2. The data are expressed as means ± S.E.M. a) P<0.05, vs H-65K$^+$. Numbers in parentheses indicate the number of muscle strips.

consumption (Table 1). Furthermore, the rate of O_2 consumption of longitudinal and circular smooth muscle increased to 0.019 ± 0.003 and 0.019 ± 0.004, in the presence of CCh (0.3 μM), respectively. The increased rate of O_2 consumption was reduced by application of NaCN, in a concentration-dependent manner (Table1).

Effects of NaCN and hypoxia on PCr and ATP contents in the presence of high K$^+$ in the porcine gastric smooth muscle

To evaluate the changes in total energy for muscle contraction in hypoxic conditions, we measured PCr and ATP contents.

In longitudinal and circular smooth muscle, hypoxia significantly reduced PCr contents in the presence of H-65K$^+$ (Table 2). Moreover, NaCN inhibited the PCr contents in a concentration-dependent manner (Table 2). Conversely, hypoxia or the addition of NaCN (1 mM) did not affect ATP content of longitudinal and circular smooth muscles (Table 2).

DISCUSSION

In anesthetized rats, acute hypoxia causes reduction in stomach pressure and increase in stomach vagal activity [10]. Thus, acute hypoxia suppresses stomach motility, and the vagus nerve is important in its regulation. However, there are no reports showing the effect of hypoxia on gastric smooth muscle contraction. This study revealed the effects on aeration of N_2 and application of NaCN on high K$^+$- and CCh-induced muscle contractions of the porcine fundus smooth muscle.

In gastrointestinal smooth muscle such as ileum [17], taenia coli [16, 21], and gallbladder, a smooth muscle [22] contraction was accompanied by an increase in oxygen consumption. In the present study, porcine longitudinal and circular muscle induced contractions with increased oxygen consumption in the presence of high K$^+$ and CCh. Our findings suggest that contractile tension in the porcine fundus smooth muscle is associated with aerobic glycolysis like in other gastrointestinal smooth muscles.

Hypoxia and NaCN significantly inhibited high K$^+$- and CCh-induced contractions in porcine gastric fundus longitudinal and circular muscles; however, the inhibition was stronger in CCh-induced contraction. Conversely, hypoxia and NaCN similarly inhibited increase in high K$^+$- and CCh-induced O_2 consumption. These results indicate that high K$^+$ and CCh contractile responses in porcine fundus smooth muscle differ in aerobic metabolic dependence.

In energy metabolism in the contraction of smooth muscle, ATP is generated from the glycolysis pathway, Tricarboxylic acid cycle, and electron transport chain, consequently phosphorylating creatine to produce PCr for energy storage. ATP required for smooth muscle contraction is supplied by the phosphorylation of ADP to ATP via creatine kinase dephosphorylation of stored PCr. With regard to smooth muscle energy metabolism, hypoxic conditions decrease muscle contraction and increase intracellular ATP and PCr contents. In the present study, we showed that hypoxia or application of NaCN inhibited high K$^+$- and CCh-induced contractions with reduces of intracellular PCr content in porcine fundus longitudinal and circular muscle. However, hypoxia or NaCN did not significantly affect ATP contents in porcine fundus smooth muscles. It has been reported that there is compartmentalization of ATP synthesis and utilization in smooth muscle [5]. Further studies will likely clarify the relationship

between muscle contraction and changes in ATP contents.

In the present study, we did not measure PCr content in the presence of CCh. Receptor agonists-induced contractions require Ca^{2+} sensitization of contractile proteins, which is primarily mediated mainly by decrease in myosin light-chain phosphatase activity, and can be regulated by protein kinase C or rho kinase [9, 18]. Thus, it is speculated that the receptor agonist induced contraction-signaling pathway requires phosphorylation, such as RhoA, PKC, and CPI-17, and requires more ATP than that of high K^+-induced contraction. However, the association of PKC and Rho K or aerobic metabolism in contraction depends on smooth muscle tissue type, and various contractile agents. In order to clarify the difference between hypoxia-induced relaxation between CCh-induced contraction and high K^+-induced contraction and the difference between longitudinal and lateral muscle of CCh-induced contraction suppression by NaCN, we will measure PCr and ATP contents in the future.

It has been reported that the ultrastructures of longitudinal and circular muscle cells are different. For example, the number of mitochondria and microtubules per unit area of smooth muscle cells is greater in the longitudinal muscle than in the circular muscle [15]. In this study, the NaCN-induced inhibition of CCh-enhanced contraction was stronger in longitudinal muscle than in circular muscle. The differences of NaCN-induced inhibition may be related to the differences of the cellular structure.

Isolated gastric fundus strips from rodents such as mouse and rat are usually used for ex vivo experiments. However, we were able to demonstrate that hypoxia and cyanide-induced inhibition of contraction in the porcine gastric fundus. Thus, our study suggests that porcine gastric fundus may be a useful tool to investigate gastric motility in acute hypoxic condition.

In summary, hypoxia and NaCN inhibited high K^+- and CCh-induced contractions in porcine gastric fundus smooth muscle by decreasing O_2 consumption and intracellular PCr content. However, the inhibition of CCh-induced muscle contraction was greater than that on high K^+-induced muscle contraction.

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