FULL PAPER *Pharmacology*

Effects of papaverine on carbachol- and high K^+-induced contraction in the bovine abomasum

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ABSTRACT. The effects of papaverine on carbachol (CCh) -and high K^+- induced contraction in the bovine abomasum were investigated. Papaverine inhibited CCh (1 μM) -and KCl (65 mM) -induced contractions in a concentration-dependent manner. Forskolin or sodium nitroprusside inhibited CCh-induced contractions in a concentration-dependent manner in association with increases in the cAMP or cGMP contents, whereas papaverine increased cGMP contents only at 30 μM. Changes in the extracellular Ca^{2+} from 1.5 mM to 7.5 mM reduced verapamil-induced relaxation in high K^+-depolarized muscles, but papaverine-induced relaxation did not change. Futhermore, papaverine (30 μM) and NaCN (300 μM) decreased the creatine phosphate contents. These results suggest that the relaxing effects of papaverine on the bovine abomasum are mainly due to the inhibition of aerobic energy metabolism.

KEY WORDS: abomasum, cAMP, cGMP, creatine phosphate, papaverine

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Based on the electrophysiological and mechanical behaviors, smooth muscles are classified as either phasic or tonic muscles [12, 19, 30]. Phasic smooth muscles are electrically quiescent into the resting condition and reveal spontaneous electrical spikes. Depolarization with high K^+ induces their initial phasic contraction, which is then followed by a decline to a low steady-state level of spike activity [11]. Phasic muscles include the ileum, urinary bladder, uterus and vas deferens. In contrast, tonic smooth muscles show depolarization with or without low-level continuous spike activities. Their high K^+-induced depolarization typically evokes a slowly developing sustained contraction as found in tonic muscles, such as the aorta and trachea. The contractile differences among different smooth muscles appear to be attributed to various pathways found in oxidative metabolism [7, 15], as well as differences in the protein expression [1, 2, 9, 20, 24, 31] and electrophysiological responses [6, 37].

The relaxing mechanism of papaverine, a non-selective smooth muscle relaxant, has been previously explained as the follows: 1) it inhibits phosphodiesterase (PDE) and results in an intracellular accumulation of cAMP and/or cGMP [22, 25, 32], 2) it inhibits mitochondrial respiration [26, 27, 33–35] and 3) it results in the effects on Ca^{2+} movement [13]. Although these different mechanisms have been proposed by respected researchers, there were no previous studies comparing and evaluating these three mechanisms in various smooth muscles. We have recently suggested that papaverine inhibited smooth muscle contraction primarily through the accumulation of cAMP and/or cGMP by inhibiting PDE in tonic muscles, such as rat aorta [16] and bovine trachea [17]. However, we found that papaverine worked to inhibit mitochondrial respiration in phasic muscles, such as guinea pig ileum [16], urinary bladder [28] and rat uterine [29]. These studies provided the first evidence that relaxation mechanisms of papaverine differ in phasic and tonic smooth muscles. In cattle, the function of abomasums, but not forestomach, is similar to that of the stomach of non-ruminant. Motility of the gastrointestinal (GI) tract, which includes abomasums in cattle, is controlled by the autonomic nervous system [4, 10]. With the exception of sphincter muscles, the motility of the GI tract is stimulated by vagal cholinergic nerves and depressed by adrenergic nerves in mammalians. However, it has been reported that the expression of adrenergic receptors mRNA in bovine GI tract is low [23]. On the other hand, the presence of nitrergic neurons in bovine GI tract has been shown by histochemical and immunofluorescence analyses [36]. However, to our knowledge, there are few reports investigating the effects of adrenergic agonists and/or nitrergic agents on the motility of bovine abomasums. Displacement of the abomasum is a common disease in dairy cattle, and one possible cause has been reported [8]. When a cow is in diagnosed with abomasum displacement, the tonus of the abomasum decreases. Therefore, examining the relaxing mechanisms of smooth muscle in the abomasum is important for understanding the pathophysiology of abomasum displacement. We applied papaverine, and the inhibitory responses are evaluated to clarify the inhibitory mechanisms in the abomasum.

In the present study, we characterized the inhibitory

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mechanisms of papaverine in the bovine abomasum (phasic muscle). This investigation was performed by measuring muscle tension and levels of cAMP, cGMP, phosphocreatine (PCr) and adenosine triphosphate (ATP) contents in the bovine abomasum.

MATERIALS AND METHODS

Muscle preparations and tension measurement: Abomasums from adult Japanese Black cattle of either sex were obtained from a local abattoir. The mucosal layers were removed by cutting with fine scissors, and smooth muscle tissues were isolated from the fundic region. The circular muscle strips were incubated with physiological salt solution (PSS) containing (in mM) 136.8 NaCl, 5.4 KCl, 1.5 $CaCl_2$, 1.0 $MgCl_2$, 23.8 $NaHCO_3$ and 5.6 glucose. PSS was aerated with 95% O_2 and 5% CO_2 to adjust the pH to 7.4 at 37°C. In some experiments, PSS included 7.5 mM $CaCl_2$. 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 2g. The muscle strips were equilibrated for 30 min to obtain a stable contractility induced by hyperosmotically added 65 mM KCl (high K^+). When the contractile response induced by 1 μM carbachol (CCh) or high K^+ reached a steady level about 15–20 min after addition to a muscle strip, papaverine, forskolin, nitroprusside, verapamil or NaCN was added cumulatively.

Assay of cAMP or cGMP content: The cAMP or cGMP content in the muscle strips was measured by enzyme immunoassay. After incubation of the strips with papaverine, forskolin or sodium nitroprusside for 10 min in the presence of CCh (1 μM), the strips were rapidly frozen in liquid nitrogen and stored at −80°C until homogenized in 6% trichloroacetic acid (0.4 m*l*). The homogenate was centrifuged at 3,000 × *g* for 15 min, and the supernatant was washed with 1.5 m*l* of water-saturated dietylether four times; the cGMP or cAMP content of the strips was assayed using an enzyme immunoassay kit (GE Healthcare, Buckinghamshire, U.K.). The cAMP or cGMP content was expressed in picomole per gram wet weight of the tissue.

Assay of PCr and ATP: PCr and ATP contents in muscle strips were measured by high-performance liquid chromatography (HPLC), as reported previously [17]. After the muscles were electrically stimulated for 5 min, papaverine or sodium cyanide (NaCN) was added to the organ bath for 20 min. After incubation, the muscles were rapidly frozen in liquid nitrogen and stored at −80°C until homogenization in 0.3 m*l* of 9% perchloric acid. The homogenate was centrifuged at 15,000 × *g* for 5 min, and then, the supernatant was neutralized with 0.25 m*l* of 2 M $KHCO_3$. Neutralized extracts were spun once more, and 20 μl of supernatant applied to HPLC.

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

Chromatography was performed using a μRPC C2/C18 ST system (4.6-mm internal diameter and 100-mm length; GE Healthcare) with mobile phase solutions of 50 mM KH_2PO_4, and 5 mM terabutylammonium hydrogen sulfate (TBAHS) (pH 6.0, buffer A), and 50 mM KH_2PO_4, 5 mM TBAHS and 40% methanol (pH 6.0, buffer B). The flow rate was 1.0 m*l*/min, and the elution started with 65% buffer A. In the first 14 min, buffer B increased at a rate of 2.5%/min. This was followed by elution with 70% buffer B for 20 min, 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 absorbance units full scale, and the oven temperature was set at 40°C. PCr and ATP contents are expressed as micromoles per gram of wet weight.

Chemicals: Chemicals used were papaverine, forskolin, sodium nitroprusside, verapamil, carbachol (Sigma-Aldrich, St. Louis, MO, U.S.A.) and NaCN (Wako Pure Chemical, Osaka, Japan). Stock solution of forskolin was prepared in ethanol, and all other drugs were prepared in distilled deionized water.

Statistics: The values are expressed as mean ± SEM, and the IC_{50} values (concentration producing 50% relaxation) were determined by linear regression analysis. Statistical analyses were performed by the Student's *t*-test. Statistical significance was established at *P*-values lower than 0.05. These calculations and statistical analysis were performed using GraphPad Prism4 and Excel 2010 for Windows.

RESULTS

Effects of papaverine, forskolin or sodium nitroprusside on the CCh- or high K^+-induced contraction: When a contractile response induced by 65 mM KCl (high K^+) or 1 μM CCh reached a steady level about 15–20 min after application, papaverine (1–100 μM) was added cumulatively. Papaverine inhibited the CCh- or high K^+-induced contraction in a concentration-dependent manner (Fig. 1A).

Forskolin (0.1–100μM), an adenylyl cyclase activator, or sodium nitroprusside (SNP), a soluble guanylyl cyclase activator (0.003–30 μM), also inhibited the CCh- or high K^+-induced contraction in a concentration-dependent manner (Fig. 1B and C). The values for IC_{50} and the maximum relaxation for these agents of CCh- or high K^+- induced contraction are presented in Table 1.

Effects of Papaverine, forskolin and SNP on cAMP and cGMP contents: In the presence of CCh (1 μM), forskolin (3 and 10 μM) increased the cAMP contents in a concentration-dependent manner, but papaverine (10 and 30 μM) did not change cAMP contents (Fig. 2A). By contrast, SNP (1 and 10 μM) increased cGMP contents in a concentration-dependent manner, but only the higher concentration of papaverine (30 μM) increased the cGMP contents, and this dose induced maximum relaxation (Fig. 2B).

Effects of extracellular calcium on papaverine- and verapamil-induced inhibitions of high K^+-induced contraction: Verapamil (0.01–30 μM) inhibited high K^+-induced contraction in a concentration-dependent manner. Increases of extracellular Ca^{2+} from 1.5 to 7.5 mM recovered the inhi-

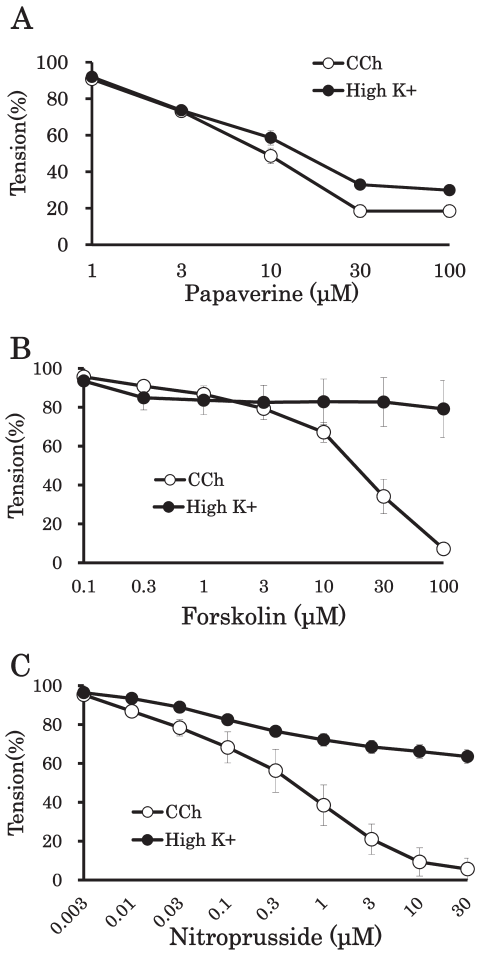

Fig. 1. Effects of papaverine, forskolin and sodium nitroprusside (SNP) on the contraction induced by 1 μM carbachol (CCh) and 65 mM KCl (high K^+) in bovine abomasum. Preparations were precontracted with CCh or high K^+, and then, the specified agents were added to the bath solution. The maximum contractions induced by high K^+ and CCh in the absence of these agents were taken as 100%. Each point represents the mean of 4–7 preparations. Vertical bars indicate SEM.

bition of high K^+-induced contraction caused by verapamil (Fig. 3B), but the same increases in extracellular Ca^{2+} did not recover the inhibition caused by papaverine (Fig. 3A).

Effects of papaverine and NaCN on a muscle contraction, and PCr and ATP contents: Figure 4A shows the effects of NaCN on CCh- or high K^+-induced contraction. NaCN (10–1,000 μM) inhibited CCh- or high K^+-induced contraction in a concentration-dependent manner.

Addition of papaverine (30 μM) and NaCN (300 μM), which showed maximum or sub-maximum inhibition of CCh-induced contraction, significantly decreased PCr, but not ATP contents in abomasum (Fig. 4B).

DISCUSSION

Based on the following observations, we have concluded that the inhibitory mechanisms of papaverine on CCh- or high K^+-induced contraction in the bovine abomasum may be due to the inhibition of mitochondrial respiration. 1) In the presence of CCh, papaverine increased cGMP content only at 30 μM and did not alter cAMP content. 2) Increases in extracellular Ca^{2+} from 1.5 to 7.5 mM attenuated the inhibition of high K^+-induced contraction caused by verapamil, but not that caused by papaverine. 3) Papaverine and NaCN decreased PCr contents in the bovine abomasum.

PDEs are currently classified into 11 families [3], and various selective PDE inhibitors have been found [5]. In our previous study, vardenafil (a selective PDE 5 inhibitor) induced relaxation in association with the increases in the cGMP content and was the most potent relaxing agent involved in inhibition of PDE5 [18]. This suggests that PDE 5 functions to hydrolyze cGMP in bovine abomasum. As reported previously, papaverine-induced relaxation was associated with increases in cAMP and/or cGMP in rat aorta [16] and bovine trachea [17]. In the present study, papaverine increased cGMP only at 30 μM, which induced maximum relaxation and did not affect cAMP content. Moreover, the papaverine-induced increases of cGMP contents were smaller than SNP-induced increases of cGMP contents. These results suggest that relaxing mechanism of papaverine may not involve with accumulation of cGMP due to the inhibition of PDE in the bovine abomasum.

Table 1. IC_{50} and maximum relaxation values for papaverine, forskolin, sodium nitroprusside and NaCN in the bovine abomasum treated with 1 μM carbachol or 65 mM KCl

Agent	Stimulant	IC_{50} (μM)	Maximum relaxation (%)	n
Papaverine	1 μM carbachol	8.1 (7.0–9.1)	81.4 ± 9.2	7
	65 mM KCl	16.5 (13.6–20.0)	73.0 ± 8.2	6
Forskolin	1 μM carbachol	12.9 (10–15.8)	88.6 ± 1.9	4
	65 mM KCl	>100	20.8 ± 11.2	6
Nitroprusside	1 μM carbachol	0.3 (0.2–0.5)	94.4 ± 5.6	6
	65 mM KCl	>30	36.5 ± 3.5	7
NaCN	1 μM carbachol	150.0 (121.3–190.1)	81.7 ± 6.1	4
	65 mM KCl	>1,000	46.7 ± 7.0	6

Numbers in parentheses indicate 95% confidence interval. The maximum relaxation represents the resting tension after washing and was considered to be 100%.

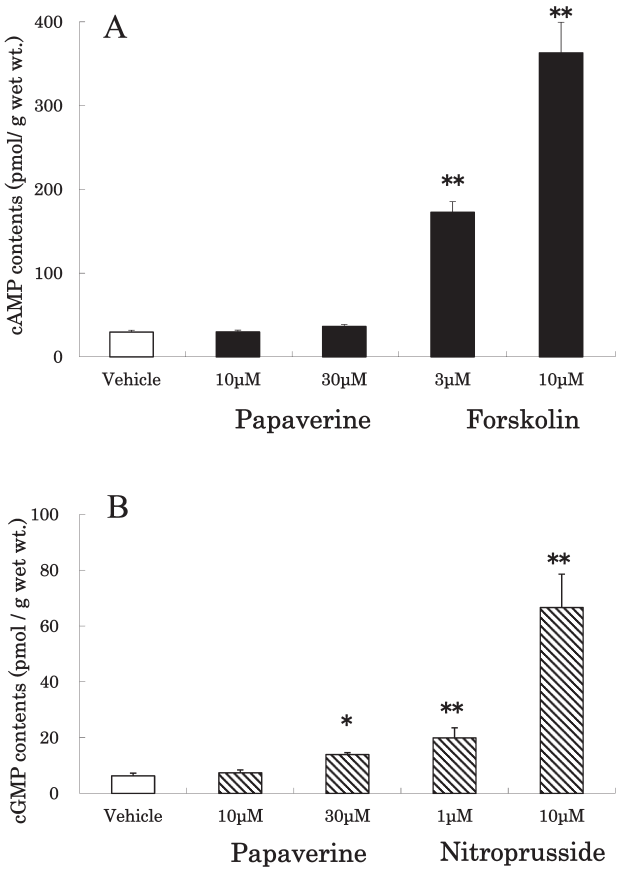

Fig. 2. The effects of papaverine and forskolin on the cAMP (A) or papaverine and SNP on the cGMP (B) contents of the bovine abomasum. Preparations were precontracted with 1 μM CCh and then treated with the specified agents for 10 min. The control was treated with vehicle instead of these agents. Each point represents the mean of 4 experiments. Vertical bars indicate the SEM. * and **: Significant difference from each respective control, with $P<0.05$ and $P<0.01$, respectively.

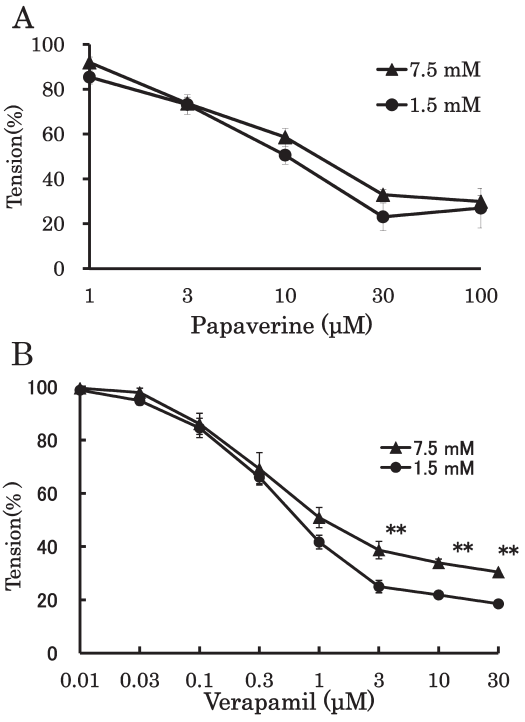

Fig. 3. The effects of extracellular Ca^{2+} on papaverine- or verapamil-induced inhibition of high K^+-enhanced contractions. Effects of papaverine (A) and verapamil (B) on contraction induced by high K^+ in abomasum in PSS including 1.5 or 7.5 mM $CaCl_2$. Ordinate: Contractions induced by high K^+ just before the application of each agent were considered as 100%. Each point represents the mean of 4–8 preparations. Vertical bars indicate SEM. **$P<0.01$ versus respective controls.

In guinea pig trachea, papaverine has been shown to inhibit a Ba^{2+} inward current in a manner independent of the intracellular cAMP levels [13]. This suggests that papaverine directly inhibits Ca^{2+} influx. In the present study, verapamil inhibited high K^+-induced contraction in the abomasum, and increasing levels of extracellular Ca^{2+} from 1.5 to 7.5 mM attenuated the inhibition of the contraction caused by verapamil. However, increases in extracellular Ca^{2+} did not affect the inhibition of high K^+-induced contraction by papaverine. These results suggest that relaxation of papaverine is not related to blockade of Ca^{2+} movement.

Some papers reported the possibility that papaverine is involved in the inhibition of mitochondrial respiration [26, 27, 33–35]. Tsuda *et al.* [33–35] showed that papaverine inhibited high K^+-induced contraction and O_2 consumption in guinea pig taenia coli. They further found that papaverine inhibited mitochondrial respiration by blocking the transduction of an electron between NADH and coenzyme Q and by inhibiting NADH, NADHP-diaphorase. Moreover, it has been thought that PCr / creatine kinase system plays a role in the transport of high energy phosphates from the mitochondrial compartment to the sites of energy utilization, correlating with oxidative metabolism in mammalian smooth muscle [15, 21]. Ishida and Takagi [14] demonstrated that papaverine decreased the content of PCr and ATP in guinea pig taenia coli in a concentration-dependent manner. In the present study, NaCN and papaverine inhibited CCh- and high K^+-induced contraction with similar potency, and papaverine and NaCN decreased PCr contents in the abomasums. On the other hand, the effects of forskolin and sodium nitroprusside were more potently CCh-induced contraction than the high K^+-induced contraction. These data indicate that the inhibitory mechanisms of papaverine on both CCh- and high K^+-induced contraction in bovine abomasum are probably similar as that in other muscles studied [28, 29]. However, in our previous study, papaverine-induced relaxation in the bovine trachea was not related to changes of PCr contents [17]. These studies suggest that the relaxing mechanism of papaverine on CCh- and high K^+-induced contraction in the bovine abomasum is closely related to the inhibition of mitochondrial respiration in guinea pig taenia coli, but not

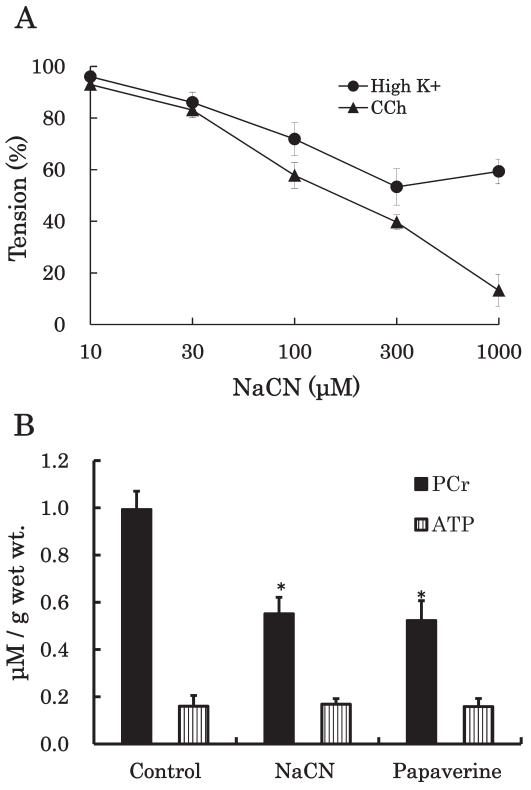

Fig. 4. The effects of NaCN on the contraction induced by CCh and high K^+ in bovine abomasum (A), and the effects of papaverine and NaCN on PCr and ATP contents (B). A: Ordinate: CCh- and high K^+-induced contractions just before the application of NaCN were considered as 100%. B: Preparations were precontracted with CCh and treated with papaverine or NaCN for 10 min. Each point represents the mean of 4–8 preparations. Vertical bars indicate SEM. *P<0.05 versus respective controls.

bovine trachea.

In many cases, abomasal displacement occurs in the early postpartum period. In the abdominal cavity, the increased uterine volume caused by pregnancy may press on nearby abdominal organs, such as the abomasum, and induce ischemic state, resulting in atony. However, we have not any data that indicate an association between the abdominal hypoxia in bovine abomasum. Further studies are needed to clarify the potential involvement of abdominal hypoxia in bovine displaced abomasum.

In conclusion, it is suggested that papaverine inhibited CCh- and high K^+-induced muscle contraction primarily via the inhibition of mitochondrial respiration in bovine abomasum which was classified as a phasic muscle.

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