FULL PAPER *Pharmacology*

PDE4 and PDE5 regulate cyclic nucleotide contents and relaxing effects on carbachol-induced contraction in the bovine abomasum

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ABSTRACT. The effects of various selective phosphodiesterase (PDE) inhibitors on carbachol (CCh)-induced contraction in the bovine abomasum were investigated. Various selective PDE inhibitors, vinpocetine (type 1), erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA, type 2), milrinone (type 3), Ro20-1724 (type 4), vardenafil (type 5), BRL-50481 (type 7) and BAY73-6691 (type 9), inhibited CCh-induced contractions in a concentration-dependent manner. Among the PDE inhibitors, Ro20-1724 and vardenafil induced more relaxation than the other inhibitors based on the data for the IC_{50} or maximum relaxation. In smooth muscle of the bovine abomasum, we showed the expression of PDE4B, 4C, 4D and 5 by RT-PCR analysis. In the presence of CCh, Ro20-1724 increased the cAMP content, but not the cGMP content. By contrast, vardenafil increased the cGMP content, but not the cAMP content. These results suggest that Ro20-1724-induced relaxation was correlated with cAMP and that vardenafil-induced relaxation was correlated with cGMP in the bovine abomasum. In conclusion, PDE4 and PDE5 are the enzymes involved in regulation of the relaxation associated with cAMP and cGMP, respectively, in the bovine abomasum.

KEY WORDS: abomasum, cAMP, cGMP, PDE inhibitor

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Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are important second messengers and have been associated with smooth muscle relaxation [7]. cAMP is synthesized by adenylyl cyclase, cGMP is synthesized by guanylyl cyclase, and both are degraded by phosphodiesterase (PDE). Currently, PDEs are classified into 11 families [3], and selective PDE inhibitors have been developed [5]. It has been reported that the relaxation induced by these selective PDE (type 1 to 5) inhibitors is involved in the increases in cAMP and/or cGMP contents in vascular [17], tracheal [19], urinary [18, 23], gastrointestinal [1, 12–14, 22] and iris smooth muscle [26]. However, to our knowledge, there have been few reports showing the effects of selective PDE7 and/or PDE9 inhibitors in smooth muscle contractions.

In cattle, the function of the abomasums, but not the forestomach, is similar to that of the stomach of the nonruminant. The motility of the gastrointestinal (GI) tract, including the abomasums in cattle, is controlled by the autonomic nervous system [4, 9]. It is well known that the motility of the GI tract, except for the sphincter muscle, is stimulated by vagal cholinergic nerves and depressed by adrenergic nerves in mammalians. However, it has been reported that the expression of mRNA of adrenergic receptors in the bovine GI tract was low [15]. On the other hand, the presence of nitrergic neurons in bovine GI tract has been shown by histochemical and immunofluorescence techniques [24]. However, to our knowledge, there are few reports to investigate the effects of adrenergic agonist and/or nitrergic agents on the motility of the bovine abomasums by pharmacological techniques.

Displacement of the abomasum is a common disease in dairy cattle. Impaired abomasal motility and an increased accumulation of gas are prerequisites for displacement of the abomasum in dairy cattle. Predisposing factors are breed, age, milk yield, genetics, nutrition, stress, metabolic disorders and neuronal disorders [8]. At the present time, the basic principle is not still known.

To date, the effect of various PDE inhibitors on muscle contraction has not yet been evaluated in bovine abomasal smooth muscle. To show the effects of various PDE inhibitors on the contraction of abomasal smooth muscle, it is thought to clear to the regulation of muscle tension by cAMP and/or cGMP which changes by PDE isozymes on physiological condition, but also pathophysiological condition, such as abomasums displacement.

In the present study, we used selective PDE (types 1–5, 7 and 9) inhibitors to examine the effects of carbachol-induced contractions of the smooth muscles in the bovine abomasums. In addition, we also investigated the expression of PDE4 and PDE5 isoforms in smooth muscles of the bovine abomasums and the changes in cAMP and cGMP contents caused by Ro20-1724 (type 4) and vardenafil (type 5).

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MATERIALS AND METHODS

Muscle preparations and tension measurement: Abomasums from adult bovine 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. Circular muscle strips were incubated with physiological salt solution (PSS) containing (in mM) 136.8 NaCl, 5.4 KCl, 2.5 $CaCl_2$, 1.0 $MgCl_2$, 23.8 $NaHCO_3$ and 5.6 glucose. The PSS was aerated with 95% O_2 and 5% CO_2 to adjust the pH to 7.4 at 37°C. 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 g. The muscle strips were equilibrated for 30 min to obtain stable contractility induced by hyperosmotic addition of 65 mM KCl. When the contractile response induced by 1 μM carbachol (CCh) reached a steady level about 15–20 min after addition to a muscle strip, vinpocetine (type 1), EHNA (type 2), milrinone (type 3), Ro20-1724 (type 4), vardenafil (type 5), BRL-50481 (type 7) or BAY736691 (type 9) was added cumulatively.

RT-PCR analysis: Total RNA was subsequently extracted from the tissues or cells with TRIzol (Invitrogen Japan, Tokyo, Japan), and then, the cells were precipitated with isopropanol and suspended to a concentration of 1 $\mu g/\mu l$ in RNase-free distilled water. RT-PCR was performed to evaluate the expression of PDEs. Briefly, first-strand cDNA was synthesized with random 6-mer oligonucleotide primers and PrimeScript Rtase (Takara Bio, Tokyo, Japan) at 30°C for 10 min, 42°C for 30 min, 95°C for 5 min and 4°C for 5 min. PCR amplification using TaKaRa Ex Taq (Takara Bio) was conducted in the presence of the oligonucleotide primers listed in Table 1. The PCR samples were amplified with 36 cycles at 94°C for 30 sec, 55°C for 45 sec and 72°C for 30 sec in a thermal cycler (Takara PCR Thermal Cycler MP, Takara Bio). The PCR products in each cycle were separated electrophoretically on a 2% agarose gel containing 0.1% ethidium bromide. FAS-III ultraviolet transilluminator (Toyobo, Tokyo, Japan) was used for visualizing the fluorescent bands.

Assay of cGMP and cAMP contents: The cGMP and cAMP contents in the muscle strips were measured by enzyme immunoassay. After incubation of the strips with Ro20-1724 or vardenafil for 10 min in the presence of carbachol (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 dietyl ether four times; the cGMP and cAMP contents of the strips were assayed by using an enzyme immunoassay kit (GE Healthcare, Buckinghamshire, U.K.). The cGMP and cAMP contents were expressed as picomoles per gram wet weight of tissue.

Chemicals: The chemicals used in the present study were forskolin, sodium nitroprusside (SNP), milrinone, BAY73-6691, carbachol (Sigma-Aldrich, St. Louis, MO, U.S.A.), vinpocetine, erythro-9-(2-hydroxy-3-nonyl) adenine·HCl (EHNA), BRL-50481 (BIOMOL Research Laboratories, Plymouth Meeting, PA, U.S.A.), Ro20-1724 (LC Laboratories, Woburn, MA, U.S.A.) and vardenafil (LKT Laboratories, St. Paul, MN, U.S.A.).

Statistics: Values are expressed as the mean ± SEM, and IC_{50} values (the concentration producing 50% relaxation) were determined by linear regression analysis. Statistical analyses were performed with the Student's *t*-test.

RESULTS

Effects of various selective PDE inhibitors on CCh-induced contraction: When a contractile response induced by 1 μM CCh reached a steady level about 15–20 min after application, each PDE inhibitor was added cumulatively. Vinpocetine (type 1), EHNA (type 2), milrinone (type 3), Ro20-1724 (type 4), vardenafil (type 5), BRL-50481 (type 7) and BAY73-6691 (type 9) inhibited the CCh-induced contraction in a concentration-dependent manner (Fig. 1). The IC_{50} and maximum relaxation values for CCh-induced contraction are presented in Table 2. The rank order of the IC_{50} values was Ro20-1724 >vardenafil >milrinone >EHNA >vinpocetine >BAY73-6691 >BRL-50481. Moreover, the rank order of maximum relaxation was vardenafil >EHNA >milrinone, Ro20-1724, BAY73-6691 > BRL-50481 >vinpocetine.

Forskolin, an adenylyl cyclase activator, or SNP, a soluble guanylyl cyclase activator, inhibited the CCh-induced contraction in a concentration-dependent manner (data not shown). The IC_{50} values for forskolin and SNP were 12.9 (10–15.8 μM) and 0.3 (0.2–0.5 μM) μM, respectively.

Expression of PDE4 and 5 in smooth muscle of the bovine abomasum: Semquantitative RT-PCR analysis using specific primers for several bovine PDE genes was performed in smooth muscle strips of the bovine abomasum to check for the expression of PDE4 (A, B, C and D) and PDE5. Positive

Table 1. Primer sequences used in the present study

Target gene	Forward primer sequence (5′ − 3′)	Reverse primer sequence (5′ − 3′)
PDE4a	TGCGGAGGTGGAGATAGAGG	TAGGAGACAGGGCAGGGATG
PDE4b	CAAGTTCCGGTGTTCTTCTCCT	ATTCCTCCATGATTCGGTCTGT
PDE4c	TTCAAGGTGGCAGAGCTAAGTG	AGCATCAGTAGGTAGGTGGCAAG
PDE4d	TGGATGAGCAGGTGGAAGAG	CACAAACGAAAGGCATGGAA
PDE5	CATACACACAAACACACACACACAC	TCTACCAGAAGCCAGAGAGAACAA
GAPDH	TTGTGATGGGCGTGAACC	CCCTCCACGATGCCAAA

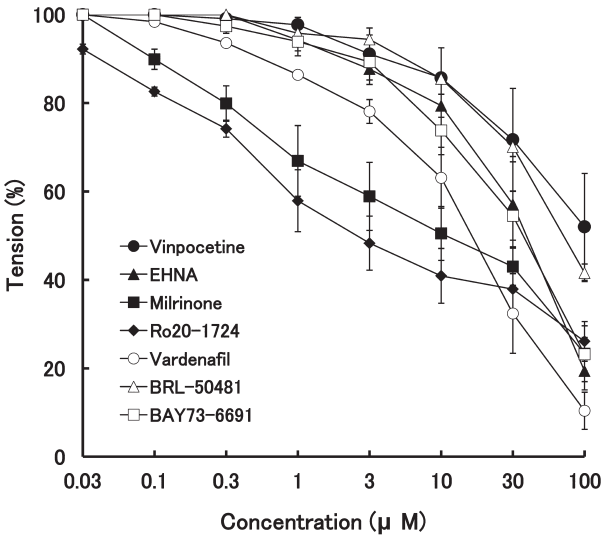

Fig. 1. Effects of vinpocetine, EHNA, milrinone, Ro20-1724, vardenafil, BRL-50481 and BAY73-6691 on the contraction induced by 1 μM carbachol (CCh) in the bovine abomasum. Preparations were precontracted with CCh, and then, the agents were added cumulatively. The maximum contractions induced by 1 μM CCh in the absence of the agents were taken as 100%. Each point represents the mean of 4-6 preparations. Vertical bars indicate the SEM.

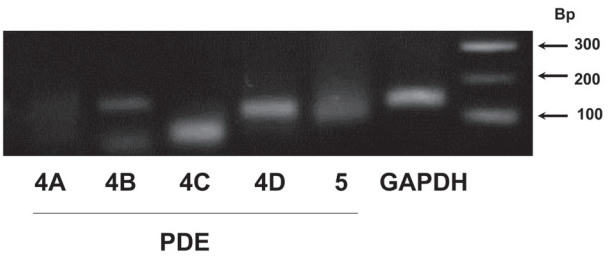

Fig. 2. Representative expression analysis of different phosphodiesterase (PDE) subtypes. Reverse transcriptase PCR analysis was performed using bovine abomasal smooth muscle. The PCR products were analyzed on 2.0% agarose gel, and pictures of the ethidium bromide-stained gels are shown. The PCR primers used for this analysis are described in Materials and Methods.

Table 2. IC_{50} and maximum relaxation values for various selective PDE inhibitors in the bovine abomasum treated with 1 μM carbachol

Agent	IC_{50} (μM)	Maximum relaxation (%)	n
Vinpocetine (type 1)	>100	48.0 ± 12.1	4
EHNA (type 2)	25.0 (15.8–31.6)	80.6 ± 4.3	6
Milrinone (type 3)	8.4 (4.5–16.0)	76.7 ± 6.3	5
Ro20–1724 (type 4)	4.3 (2.5–7.9)	73.9 ± 4.5	5
Vardenafil (type 5)	10.0 (7.9–12.6)	89.6 ± 4.2	4
BRL-50481 (type 7)	79.0 (63.1−100.0)	58.4 ± 2.0	4
BAY73-6691 (type 9)	25.0 (20.0−32.0)	76.8 ± 3.8	4

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

controls were performed using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Fig. 2). Bovine abomasal smooth muscle expresses PDE4B, PDE4C, PDE4D and PDE5. Regarding PDE5, two products with different sizes of PDE5 were amplified, indicating the existence of two splice variants (Fig. 2).

Effects of Ro20-1724 and vardenafil on cGMP and cAMP contents: In the presence of CCh (1 μM), Ro20-1724 (10 and 100 μM) increased the cAMP content in a concentration-dependent manner (Fig. 3A), but did not affect the cGMP content (data not shown). By contrast, vardenafil (30 and 100 μM) increased the cGMP content (Fig. 3B), but did not affect the cAMP content (data not shown).

DISCUSSION

Physiological abomasal contractions are regulated by second messengers, such as calcium ion, cAMP and cGMP. In the bovine abomasum, forskolin and SNP inhibited the muscarinic agonist carbachol (CCh)-induced contraction in a concentration-dependent manner. These results suggest that the cAMP/adenyl cyclase pathway and cGMP/guanylyl cyclase pathway are both involved in the relaxation of bovine abomasal smooth muscle. Therefore, we examined the effects of selective PDE inhibitors on CCh-induced contraction to confirm the role of cAMP and cGMP in mediation of abomasal contractions.

PDE regulates the intracellular concentrations of cAMP and cGMP and is classified into 11 families [3]. Moreover, the PDE1-5 isozymes have selective inhibitors [5], and selective inhibitors for PDE7 and 9 have also been made recently and commercialized. It has been shown that the potency of the relaxation induced by these PDE inhibitors differed in many smooth muscles including gastrointestinal smooth muscles [1, 12–14, 22]. Therefore, we investigated the effects of selective PDE inhibitors for PDE1-5, 7 or 9 on CCh-induced contractions of the smooth muscles in the bovine abomasum.

It has been reported that selective inhibitors for PDE1-5 inhibited agonist-induced contraction dose-dependently, that rolipram (type 4) was most effective in the guinea pig trachea [19] and that zaprinast (type 5) was most effective in the rat urinary bladder [18] and porcine iris sphincter muscle [26]. On the other hand, selective inhibitors for PDE1-5 also inhibited agonist-induced contraction dose-dependently in gastrointestinal smooth muscle, zaprinast (type 5) was most effective in the guinea pig taenia coli [13] and ileum [12], and Ro20-1724 (type 4) was most effective in the guinea pig gall bladder [14]. In the present study, selective PDE inhibitors inhibited CCh-induced contraction in the bovine abomasum, dose-dependently, and Ro20-1724 (type 4) and vardenafil (type 5) were more effective than the other PDE inhibitors. Since the rank order of inhibitory potency was different by organ, it is suggest that the characterization of

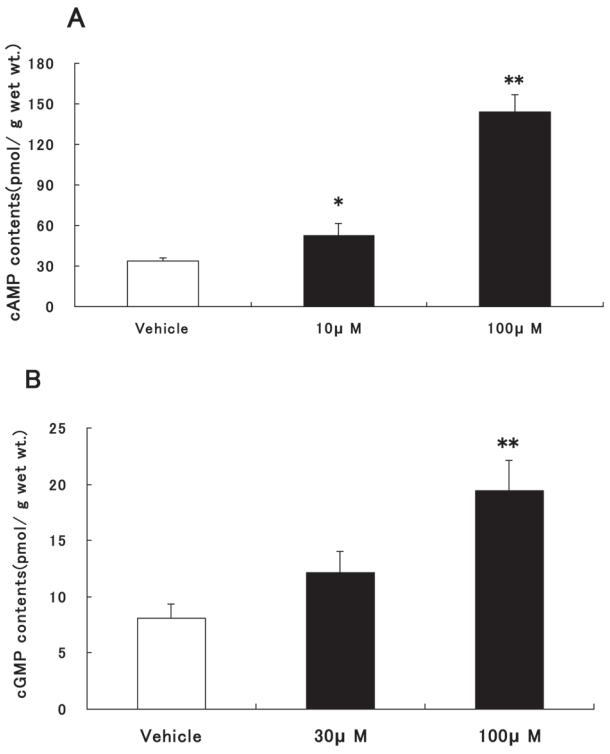

Fig. 3. The effects of Ro20-1724 (A) and vardenafil (B) on the cAMP or cGMP contents of the bovine abomasum. Preparations were precontracted with 1 μM CCh and were then treated with the agents for 10 min. The control was treated with vehicle instead of the PDE inhibitors. Each point represents the mean of 4-5 experiments. Vertical bars indicate the SEM. * and **: Significant difference from each respective control, with $P<0.05$ and $P<0.01$, respectively.

PDE isozymes differs by organ. Moreover, it is clear that the types 4 and 5 inhibitors were more potent than the other types of inhibitors in bovine abomasum.

It is well known that PDE4 is specific for the hydrolysis of cAMP [2]. Moreover, it is now recognized that there are four genes (PDE4A-PDE4D) that make up the PDE4 family, and PDE4 is expressed in a plethora of tissues and cell types and plays a role in a large number of physiological processes. In the present study, we showed the expression of PDE 4B, 4C and 4D in bovine abomasal smooth muscles by RT-PCR methods. It has been reported that rolipram (type 4) potently relaxed electrical field stimulation-induced contraction in the cat gastric fundus [1]. Ro20-1724 inhibited CCh-induced contraction in the bovine abomasum (in the present study) and guinea pig gall bladder [14] potently, but poorly in the guinea pig ileum [12] and taenia coli [13]. These data suggested that the functional distributions of PDE4 isozymes of gastrointestinal smooth muscles differ by organ.

In our study, the relaxation effect of vardenafil was more potent than those of the other cGMP-related PDE inhibitors, and vardenafil increased the cGMP content. These data are consistent with the report that vardenafil also inhibited phenylephrine-induced contraction in the rat aorta and resulted in an increase in cGMP content [21]. Moreover, zaprinast, a PDE5 inhibitor, relaxed the guinea pig taenia coli and ileum the increases in cGMP content [12, 13]. In the present study, we showed the expression of PDE5 in bovine abomasal smooth muscles by RT-PCR methods. The data suggested that the PDE5 isozymes have an important role in regulation of motility in gastrointestinal smooth muscles.

PDE7 is a cAMP-specific enzyme, and two PDE genes (PDE7A and B) have been identified. PDE7A is expressed in a number of cell types including muscle cells [3]. In the trachea of ovalbumin-sensitized guinea pig, but not in healthy guinea pig trachea, BRL-50481 inhibited histamine-induced contraction [16]. Moreover, the inhibitory effects of BRL-50481 were poor than those of the other PDE inhibitors we used for bovine abomasal smooth muscles. Thus, it is probable that the PDE7 expression in smooth muscle is increased by inflammatory disease, but in normal condition, the expression of PDE7 is poor and modifies smooth muscle tension less potently than the other PDEs in various tissues including the bovine abomasum.

PDE9 has the highest affinity for cGMP and is similar to PDE5 [3]. PDE9A is the only isoform identified, and PDE 9A is expressed in all tissues, except the blood in mammals [11, 20]. Similar manner of expression was detected for PDE9A and PDE5 mRNAs in the mouse corpus cavernosum; however, BAY 73-6691 on its own did not relax the mouse corpus cavernosum or alter sildenafil (type 5)-induced relaxation [6]. In bovine abomasums, we did not compare expression of PDE5 and 9; however, the IC_{50} for CCh-induced contraction of BAY73-6691 is 2.5 times lower than that of vardenafil. BAY 73-6691 preferentially inhibits PDE9 more than PDE 5, over 77 times. [25]. These data raise the possibility that the PDE9 is expressed in smooth muscles including the bovine abomasum. However, we infer that the regulation of normal smooth muscle contraction performed by PDE9 may be less than that of PDE5 in bovine abomasum.

It has been reported that the contraction of preparations originating from the abomasal antrum of a healthy cow evoked by electrical stimulation was inhibited by left displaced abomasum (LDA), right displaced abomasum (RDA) or abomasal volvulus (AD). These inhibitions were reversed in the presence of L-NAME [10]. These data imply that the expression of nitric oxide (NO)-cGMP signals, including the expression of PDEs, may increase in the abomasum of LDA, RDA or AV. However, we have no data indicating the changes in expression of PDEs in the bovine abomasum affected by these condition. A further study is involvement of the changes in the expression of PDEs in the bovine displaced abomasum.

In conclusion, selective PDE inhibitors inhibited CCh-induced contraction in the bovine abomasum. Among the PDE inhibitors, Ro20-1724 and vardenafil induced more relaxation than the other inhibitors according to the data for the IC_{50} and maximum relaxation. Moreover, the results suggest that Ro20-1724-induced relaxation was correlated with cAMP and that vardenafil-induced relaxation was correlated

with cGMP in the bovine abomasum. PDE4 and PDE5 are the enzymes involved in the regulation of relaxation associated with cAMP and cGMP, respectively, in the bovine abomasum.

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