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Involvement of Spontaneously Formed Cyclic Nucleotides in Cat Gastric Muscle Relaxation

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Muscle strips and muscle cells from cat stomach were used to investigate whether spontaneously formed cyclic nucleotides were involved in the inhibition of gastric smooth muscle contraction. A phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX), increased the levels of both cyclic GMP (cGMP) and cyclic AMP (cAMP) in resting state cells, while decreasing acetylcholine-induced muscle contraction. Under the influence of IBMX, SQ22536, an adenylyl cyclase inhibitor and methylene blue, a guanylyl cyclase inhibitor completely blocked increases in cAMP and cGMP respectively, without any effect on contraction. However, the combination of SQ22536 and methylene blue completely blocked increases in both cAMP and cGMP levels and stimulated contractions markedly even in the presence of IBMX. Muscle contraction inhibitors such as isoprenaline, vasoactive intestinal polypeptide and sodium nitroprusside also appeared to increase cyclic nucleotide levels which decreased contraction. Which nucleotide increased the most was dependent on the agonist used. Therefore, irrespective of the cyclic nucleotide class, the spontaneous formation of cyclic nucleotides should be considered in evaluating the mechanism of gastric smooth muscle relaxation.

Key Words: Smooth muscle relaxation, Cyclic AMP, Cyclic GMP, Cat stomach

INTRODUCTION

Receptive relaxation is known to be mediated via inhibitory non-adrenergic, non-cholinergic neurons of the vagus nerve, which aids the stomach to accommodate large volumes of food with little if any increase in pressure (Abrahamsson & Jansson, 1969; Takahashi & Owyang, 1995). Nitric oxide (NO) and vasoactive intestinal polypeptide (VIP) have been proposed to be non-adrenergic, non-cholinergic neurotransmitters involved in the relaxation of gastrointestinal muscle (Costa et al, 1986; Manzini et al, 1986). The immunoreactivity of VIP or NO synthase was detected in intrinsic neurons of the stomach (Fahrenkrug et al, 1978; Bredt et al, 1990; Costa et al, 1992). Consistent with this, non-adrenergic-, non-cholinergic-induced relaxation with high K^+ concentration or electrical stimulation was inhibited by VIP antagonists or NO synthase inhibitors (D'Amato et al, 1988; Li & Rand, 1990; Boeckxstaens et al, 1991; Shimamura et al, 1993). The inhibitory actions of VIP and NO for contraction is likely to be due to an increase in cellular levels of cyclic nucleotides, since VIP increases the levels of adenosine 3',5'-cyclic monophosphate (cAMP) and guanosine 3',5'-cyclic monophosphate (cGMP), and NO increases the level of cGMP in gastric smooth muscle (Torphy et al, 1986; Chakder & Rattan, 1993; Jin et al, 1993).

In cat gastric muscle strips, we have observed that a nonspecific phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) concentration-dependently inhibited acetylcholine-induced contraction without stimulating the non-adrenergic, non-cholinergic neurons (Hong & Kim, 1995). Furthermore, Kaneda et al (1997) recently reported that IBMX increased

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cGMP to inhibit the contraction of guinea pig ileal longitudinal muscle in the presence of high K^+ or carbachol. However, the role of spontaneously formed cyclic nucleotides in gastric smooth muscle cells in the control of muscle tension is not well understood.

Therefore, the aim of the present study was to investigate the involvement of the spontaneous production of cyclic nucleotides, cAMP and cGMP, in gastric smooth muscle relaxation. For this purpose, IBMX in the presence of or not in the presence of adenylyl and guanylyl cyclase inhibitors was employed and the changes in the tension of muscle strips and in the cAMP and cGMP level of muscle cells were measured. The role of the cyclic nucleotides stimulated by muscle relaxation agents which inhibit cat gastric muscle contraction was also investigated.

METHODS

Materials

Cyclic nucleotide assay systems ([^{125}I]cAMP and [^{125}I]cGMP Kits) were obtained from Amersham (Bucks, UK). Acetylcholine bromide, a nonspecific phosphodiesterase inhibitor (3-isobutyl-1-methylxanthine, IBMX), an adenylyl cyclase inhibitor (SQ22536), a guanylyl cyclase inhibitor and NO scavenger (methylene blue), NO donor (sodium nitroprusside), β-adrenergic agonist (isoprenaline), VIP, VIP antagonists ([D-p-Cl-Phe^6,Leu^{17}]-VIP and [Lys^1,Pro^{2,5},Arg^{3,4}, Tyr^6]-VIP), NO synthase inhibitors (N^G-nitro-L-arginine and N^G-nitro-L-arginine methyl ester), and β-adrenergic antagonists (propranolol and alprenolol) were from Sigma (St. Louis, MO, USA).

Preparation of gastric smooth muscle strips

Cats of either sex (2.0~3.0 kg) were anesthetized with 20% urethane (5 ml/kg, intraperitoneal) following 16 h of fasting but with water *ad libitum*. The whole stomach was removed from each cat, and the mucous membrane was peeled off in ice-cold Krebs bicarbonate solution (mM: 120.8 NaCl, 4.5 KCl, 15.5 $NaHCO_3$, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4 and 5.6 dextrose). The Krebs bicarbonate solution was aerated with 95% O_2-5% CO_2 until the pH was 7.4. Circular muscle strips (1.0×0.2 cm) were prepared from the fundus, cutting at a right-angle to the greater curvature (Sim et al, 1997).

Measurement of contractile response

The circular muscle strips were used to measure the contraction in a cylinder-shaped muscle chamber (10 ml capacity) filled with Krebs bicarbonate solution. The solution of the chamber was kept at 36°C and was bubbled with a mixture of 95% O_2-5% CO_2 at pH 7.4. To record the isometric contraction, the lower end of the muscle preparation was anchored to a steel hook and the upper end to a force transducer (FT03, Grass Instruments Co., Quincy, MA, USA) connected to a Grass 7E polygraph. The preparation was loaded with a tension of 2 g and allowed to equilibrate with the solution for 30 min unless noted otherwise. The final concentrations of agonists, antagonists or inhibitors used were achieved by adding 10 μl to the chamber. Antagonists and/or inhibitors were administered 4 min before the treatment with IBMX. Four min after the treatment with IBMX, 1 μM acetylcholine was added and isometric contraction was measured. Contractile responses to acetylcholine were recorded for 10 min. The maximal amplitude was taken as the contraction measurement for each case.

Preparation of dispersed gastric smooth muscle cells

To investigate whether the contractile responses of muscle strips were influenced by increases in cellular levels of cyclic nucleotides, muscle cells were isolated from muscle strips of cat stomach as described previously (Collins & Gardner, 1982; Sim et al, 1993). Briefly, muscle strips were dissected with a tissue slicer (Thomas Co., Philadelphia, PA, USA) into 0.5 mm thickness and were then digested with 0.3% collagenase, 0.3% papain and 0.03% soybean trypsin inhibitor. The cells were harvested by filtration through 500 μm Nitex and then washed three times with 30 ml of enzyme-free Krebs bicarbonate solution. The cells were resuspended in an adequate volume of Krebs bicarbonate solution containing 20 mM Na-Hepes (pH 7.4) to measure the levels of cyclic nucleotides.

Measurement of cAMP and cGMP

In order to examine the effects of inhibitory neurotransmitters, SQ22536 or methylene blue as well as IBMX on the cellular levels of cyclic nucleotides in resting state, cAMP and cGMP were measured by radioimmunoassay. Inhibitory neurotransmitters, SQ22536

or methylene blue was added to 0.8 ml of cell suspension in the presence or absence of 30 μM IBMX. The reaction was terminated after 4 min with 65% ethanol. Supernatant was transferred into a glass tube and the remaining precipitate was washed with ice cold 65% ethanol. The combined supernatant was centrifuged at 2,000 *g* for 15 min at 4°C and then evaporated under a stream of nitrogen at 60°C. The samples were reconstituted for radioimmunoassay in 500 μl of 50 mM Na acetate (pH 6.2) and acetylated with triethylamine/acetic anhydride (3 : 1 vol/vol) for 30 min. cAMP and cGMP were measured in duplicate using 100 μl aliquots. The measurements were expressed as pmol per mg protein.

Statistical analysis

The results are represented as means±SD and analyzed statistically by the Newman-Keuls test and regression analysis. Statistical significance was established at $P<0.05$.

RESULTS

Effect of IBMX on acetylcholine-induced contraction

Upon treatment of gastric smooth muscle with 1 μM acetylcholine, a prompt contractile response was observed. As shown in Fig. 1, the acetylcholine-induced contraction was concentration-dependently inhibited by pretreatment of the muscle strips with IBMX at concentrations ranging between 10 μM and 1 mM for 4 min. This result implies that an inhibitory effect may be attributable to the accumulation of cyclic nucleotides resulting from the inhibition of phosphodiesterase activities by IBMX, a nonspecific phosphodiesterase inhibitor. We examined whether the inhibitory effect of IBMX was dependent on the load applied to the muscle strips for recording isometric contraction, because stretch could be an adequate stimulus for the smooth muscle to activate various signal transduction systems. As the initial load increased, the contractile response to acetylcholine significantly increased (Fig. 2A). However, the inhibitory effect of IBMX on the contraction was not altered despite the increase in initial load ranging from 0.5 g to 4.0 g (Fig. 2B).

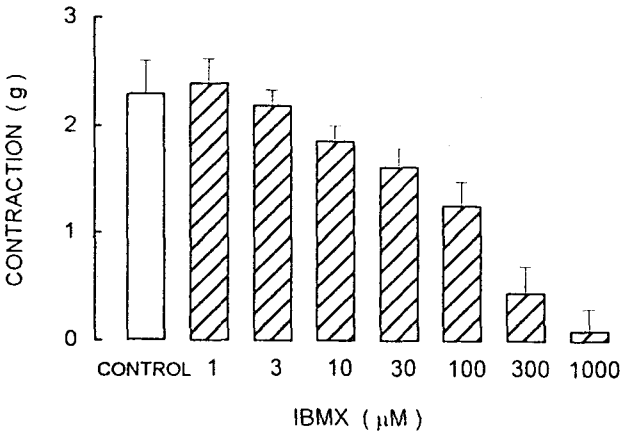

Fig. 1. Inhibitory effect of IBMX on acetylcholine-induced contraction of gastric smooth muscles. Muscle strips were incubated with various concentrations of IBMX indicated for 4 min under 2.0 g tension, and then the isometric contraction was recorded after addition of 1 μM acetylcholine. IBMX significantly decreased the acetylcholine-induced contraction in a concentration-dependent manner ($r=0.874$, $df=38$, $P<0.01$). Results are mean±SD from 8 experiments.

Effects of SQ22536, methylene blue and inhibitory agonists on acetylcholine-induced contraction

In the absence of IBMX, 10 μM SQ22536, an adenylyl cyclase inhibitor, and 1 μM methylene blue, a guanylyl cyclase inhibitor, significantly increased the contractile response to acetylcholine, and moreover, combined treatment with SQ22536 and methylene blue showed an additive effect which is comparable to an algebraic sum of each incremental effect. In contrast, several inhibitory agents, such as isoprenaline (10 μM), VIP (100 nM) and sodium nitroprusside (1 μM) significantly inhibited the contraction (Fig. 3).

Effects of contraction inhibitory agents on IBMX-treated contraction and cyclic nucleotides level

While significantly inhibiting acetylcholine-induced contraction, 30 μM IBMX significantly increased both cAMP and cGMP levels as compared to control levels (cAMP: 0.90±0.16 pmol/mg protein to 1.59±0.19 pmol/mg protein; cGMP: 0.05±0.01 pmol/mg protein to 0.07±0.01 pmol/mg protein) (Fig. 4). The inhibitory agents, isoprenaline (10 μM), VIP (100 nM) and sodium nitroprusside (1 μM), significantly inhibited the acetylcholine-induced contraction in the

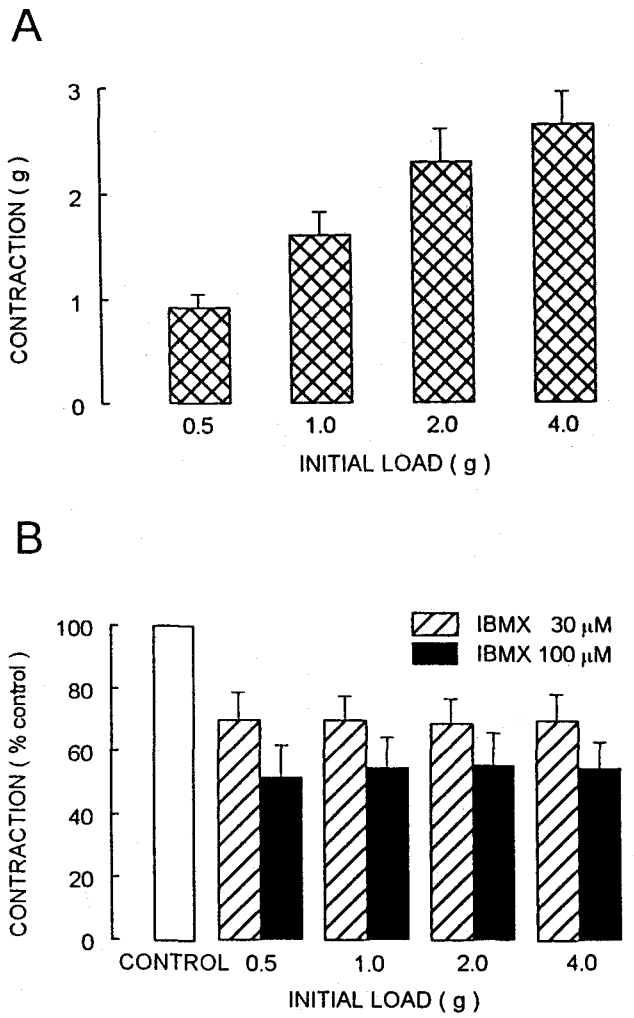

Fig. 2. Effect of initial load on acetylcholine-induced isometric contraction of gastric smooth muscle. (A) The gastric muscle strips were loaded with 0.5, 1.0, 2.0 and 4.0 g and the contractions in response to acetylcholine (1 μM) were recorded, respectively. The contraction increased significantly with increase in initial load ($r=0.779$, $df=30$, $P<0.01$). (B) Relaxant effect of IBMX on acetylcholine-induced contraction under various load conditions. Data are presented as a percentage of contraction value (control) of each initial load. The contractions were significantly decreased by pretreatment with IBMX, but the inhibitory effect of IBMX did not appear to be related to the increases in initial load. Results indicate mean $\pm$ D from 8 experiments.

presence of IBMX as compared with that of control with or without IBMX (Fig. 4A). Moreover, the effects of the contraction inhibitory agents combined with IBMX were more prominent than those of the inhibitory agents without IBMX (Figs. 3 & 4). The levels of cyclic nucleotides were significantly increased by these agents; isoprenaline significantly increased the level of cAMP, VIP increased both cAMP and cGMP, and sodium nitroprusside increased cGMP (Fig. 4B & C). However, β-adrenergic and VIP antagonists and NO synthase inhibitors, when administered in combination, did not have any influence on the inhibited contraction induced by IBMX (Fig. 5). Thus, the inhibitory effect of IBMX on the contraction of gastric muscle strip induced by acetylcholine could be inferred to be associated with the increase in both cAMP and cGMP levels of the muscle cells rather than with the release of the supposed contraction inhibitory agents.

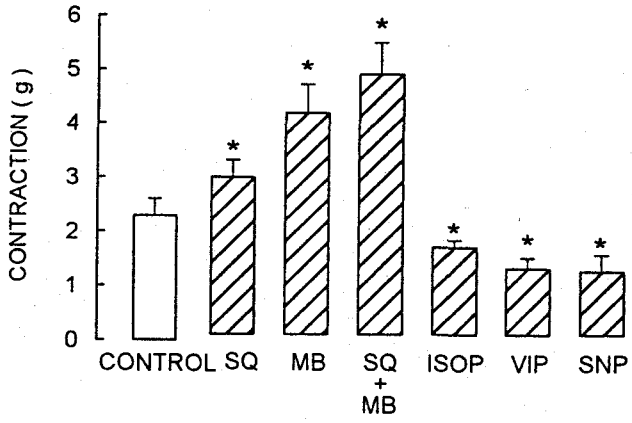

Fig. 3. Effects of adenylyl and guanylyl cyclases inhibitors and contraction inhibitory agents on the contraction of gastric smooth muscles. The muscle strips were incubated with SQ22536 (SQ, 10 μM) and/or methylene blue (MB, 1 μM) for 4 min and then the isometric contractions were recorded after addition of 1 μM acetylcholine. Contraction inhibitory agents, isoprenaline (ISOP, 10 μM), vasoactive intestinal polypeptide (VIP, 100 nM) and sodium nitroprusside (SNP, 1 μM), were also added in the same way. Results are mean $\pm$ SD from 8 experiments. *$P<0.05$ vs. control.

Effects of SQ22536 and methylene blue on IBMX-treated contraction and cyclic nucleotides levels

Pretreatments with SQ22536 and methylene blue, also significantly inhibited the acetylcholine-induced contractions even in the presence of IBMX, and their inhibitory effects were not different from that of IBMX alone (Fig. 6A). Since SQ22536 and methylene blue completely blocked the increases in cAMP level and in cGMP level, respectively, this result suggests that an increase in the level of either cAMP or cGMP is sufficient to inhibit contraction (Fig. 6B

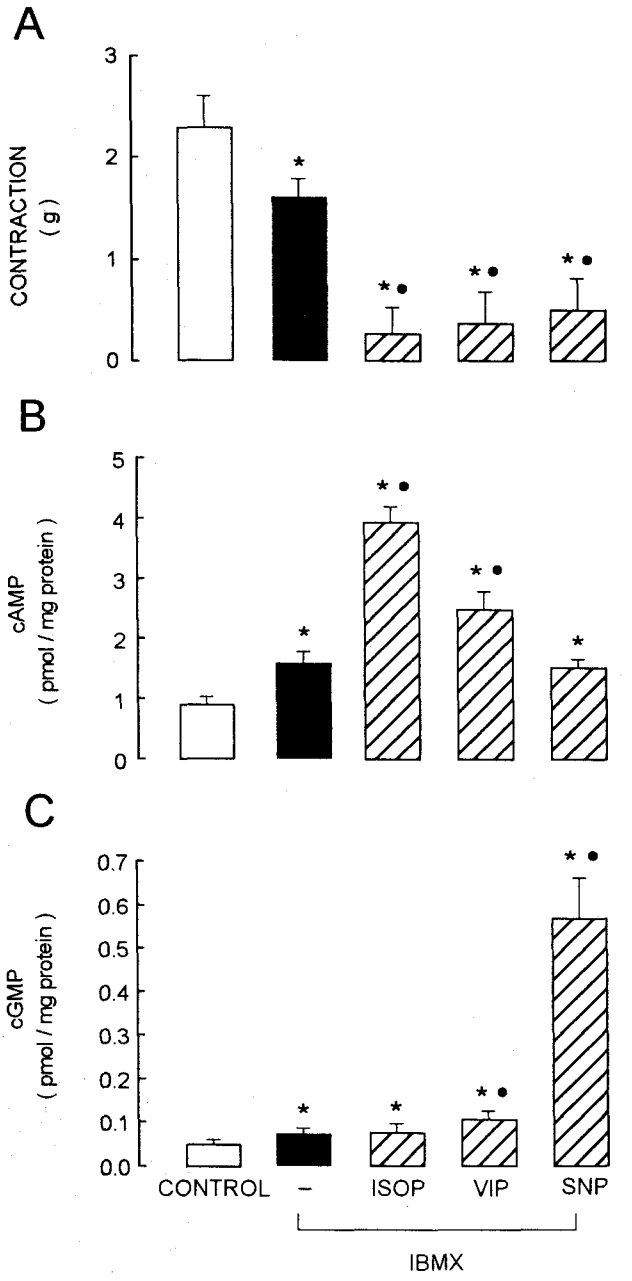

Fig. 4. Effects of contraction inhibitory agents on the acetylcholine-induced contraction and cyclic nucleotide levels of gastric smooth muscle. (A) The gastric muscle strips were incubated with isoprenaline (ISOP, 10 μM), vasoactive intestinal polypeptide (VIP, 100 nM) and sodium nitroprusside (SNP, 1 μM) for 4 min prior to adding IBMX (30 μM), and then the isometric contractions were recorded as in Fig. 1. Results are mean$\pm$SD from 8 separate experiments. (B & C) Isolated gastric muscle cells were incubated with the inhibitory agents for 4 min prior to incubation with IBMX (30 μM). The concentrations of cAMP and cGMP were measured as in Fig. 4. Results indicate mean$\pm$SD from 4 separate experiments. *$P<0.01$ vs. control; •$P<0.05$ vs. IBMX alone.

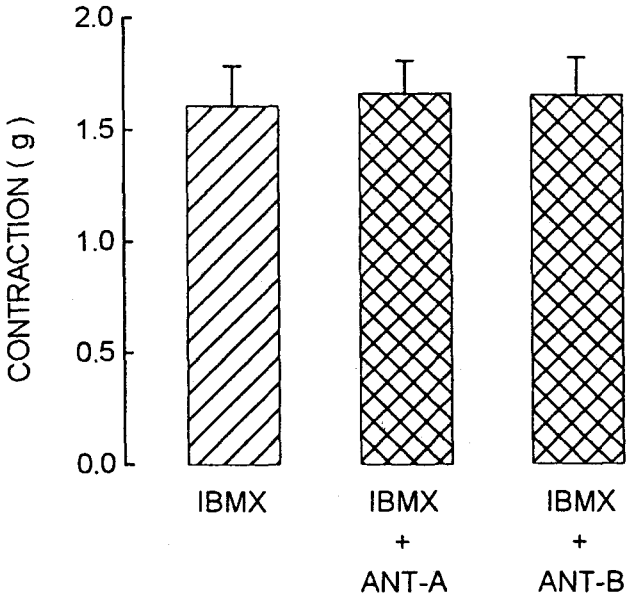

Fig. 5. Effects of β-adrenergic and VIP antagonists and NO synthase inhibitors on the inhibitory effects of IBMX in acetylcholine-induced contraction. Incubation of gastric muscles were carried out for 4 min with the antagonists or inhibitors and continued for another 4 min in the presence of 30 μM IBMX. Isometric contractions were induced by 1 μM acetylcholine. ANT-A: [D-p-Cl-Phe^6, Leu^{17}]-VIP (100 nM)+N^G-nitro-L-arginine (1 mM)+propranolol (10 μM), ANT-B: [Lys^1,Pro^{2,5},Arg^{3,4},Tyr^6]-VIP (100 nM)+N^G-nitro-L-arginine methyl ester (1 mM)+alprenalol (10 μM). Results are mean$\pm$SD from 8 experiments.

& C). However, the combination of SQ22536 and methylene blue markedly increased contraction, with the magnitude of the contraction being even greater than that of the control free of IBMX (Fig. 6A). Concomitantly, the incremental effect of IBMX on the levels of cAMP and cGMP was completely blocked (Fig. 6B & C). The markedly increased contraction in response to combined treatment of these inhibitors, thus, appears to result from the concomitant decrease of these two cyclic nucleotides production, notwithstanding the presence of IBMX.

DISCUSSION

In our isometric muscle contraction measuring system, the amplitude of acetylcholine-induced contraction was found to be dependent on the force loaded initially. According to this observation, we made the assumption that the cellular signal transduction sys-

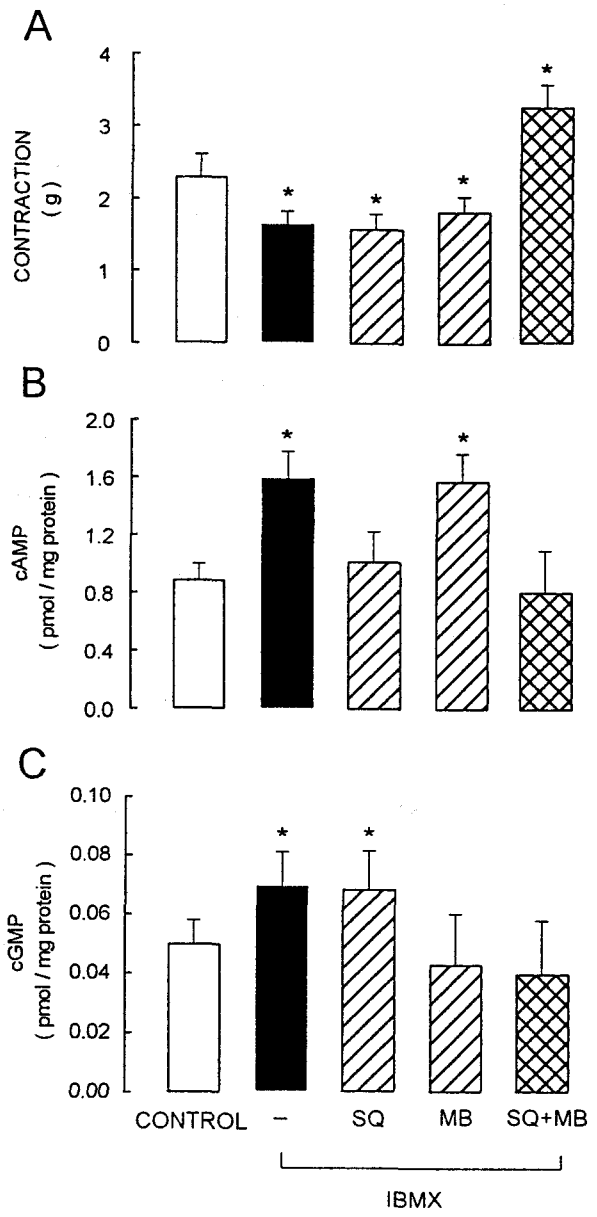

Fig. 6. Effects of SQ22536 (SQ) and methylene blue (MB) on the contraction and on the cyclic nucleotides level in the presence of IBMX. (A) Gastric muscle strips were incubated with SQ22536 (SQ, 10 μM) and/or methylene blue (MB, 1 μM) for 4 min prior to adding IBMX (30 μM), and then the isometric contractions were recorded after addition of 1 μM acetylcholine as in Fig. 1. Results indicate mean$\pm$SD from 8 experiments. (B & C) Isolated gastric muscle cells were incubated with SQ22536 and/or MB for 4 min prior to incubation with IBMX (30 μM). The reactions were terminated by adding ice-cold absolute ethanol (65% of final concentration). The concentrations of cAMP and cGMP in each sample were measured by radioimmunoassay. Results indicate mean$\pm$SD from 4 separate experiments. *$P<$ 0.01 vs. control.

tem including cyclic nucleotides might be altered by stretching the muscle. As shown in the results of Fig. 2, the inhibitory effects of IBMX, a nonspecific phosphodiesterase inhibitor, on contraction were nearly constant regardless of increasing load, implying that the initial tension applied had no relationship with the cyclic nucleotides level of cat gastric muscle. Similar results were also reported from cardiac myocytes (Sadoshima & Izumo, 1993).

In the present study, IBMX concentration-dependently inhibited the muscle contraction induced by acetylcholine. It may be possible that the inhibitory effect of IBMX on the acetylcholine-induced contraction could be mediated through the release of inhibitory neurotransmitters from the myenteric plexus of stomach muscle strips. Recently, Izzo et al (1997) reported that the inhibitory effect of a phosphodiesterase inhibitor, papaverine, on the contraction induced by electric field stimulation partly involved the release of noradrenaline from sympathetic nerves in the guinea-pig ileum. But the inhibitory effects of IBMX on the contraction were still preserved, even after the antagonists and inhibitors for the inhibitory transmitters supposedly involved were added. Therefore, the release of inhibitory transmitters does not seem to be involved in decreased muscle contraction in response to IBMX. Instead, the inhibitory effect of IBMX on the gastric muscle contraction appears to be associated with events occurring in muscle cells. IBMX significantly increased the levels of cAMP and cGMP in cat gastric muscle cells in the resting state. The results of the present study are mostly consistent with those obtained from the longitudinal smooth muscle of guinea pig ileum (Kaneda et al, 1997). Therefore, it appears that the inhibitory effect of IBMX is associated with the increased level of these cyclic nucleotides, as long as IBMX inhibits the hydrolysis of the spontaneously occurring nucleotides.

SQ22536, an adenylyl cyclase inhibitor or methylene blue, a guanylyl cyclase inhibitor significantly increased acetylcholine-induced contraction in the absence of IBMX. Moreover, when these two cyclase inhibitors were treated together, the contraction was even greater. These results suggest that cyclic nucleotides are spontaneously produced in resting gastric muscle cells. However, SQ22536 or methylene blue failed to increase contraction under the influence of IBMX. When combined, these inhibitors synergistically stimulated muscle contraction. Considering that SQ22536 and methylene blue blocked the increase in

cAMP and cGMP, respectively, the increase of cellular levels of either cAMP or cGMP seems to be sufficient to inhibit contraction as long as it remains above a certain level owing to IBMX (Kaneda et al, 1997).

Combined treatment of SQ22536 and methylene blue completely eliminated the effects of IBMX on the level of cAMP and cGMP, and contraction was markedly increased above the levels of controls with or without IBMX. This finding provides further evidence that gastric muscle cells produce both cAMP and cGMP spontaneously, otherwise the combination of SQ22536 and methylene blue would not have increased the contraction that much. Although the cyclic nucleotides levels in response to SQ22536 combined with methylene blue in the presence of IBMX were not different from those of the control cells free of IBMX, the contraction responses to the inhibitors were significantly greater than that of the control. It is, therefore, inferred from the above results that a succession of spontaneous cyclic nucleotide formations and breakdowns may play an important role in controlling gastric muscle tension. However, the mechanism and the extent by which the spontaneously formed cyclic nucleotides participate in the regulation of muscle tension needs further studies.

Contraction inhibitory transmitters such as VIP and NO have been reported to be released from the enteric nervous system by high K^+ or electrical stimulation, by which the gastrointestinal tract is relaxed (Costa et al, 1986; Manzini et al, 1986). In our cat stomach muscle study, treatments with isoprenaline, VIP and sodium nitroprusside significantly decreased the acetylecholine-induced contraction. Although these transmitters are not released by IBMX, their inhibitory effects were especially greater under the influence of IBMX, which was accompanied with significant increases in cAMP and/or cGMP levels, implying that even the inhibitory effects of inhibitory agents on the contractions, at least in part, resulted from elevation of cyclic nucleotides levels (Kwon et al, 1993).

In conclusion, we have demonstrated that IBMX concentration-dependently inhibited acetylcholine-induced contraction, which is accompanied with an increase in cellular cyclic nucleotide levels in cat gastric muscle. Under the influence of IBMX, combined treatment with SQ22536 and methylene blue restored the cyclic nucleotides to the level of control free of IBMX, and markedly stimulated contraction. Therefore, it is strongly suggested that spontaneously formed cyclic nucleotides possibly play an important role in the relaxation of cat gastric muscle.

ACKNOWLEDGMENT

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