Effects of Various Selective Phosphodiesterase Inhibitors on Muscle Contractility in Guinea Pig Ileal Longitudinal Smooth Muscle

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ABSTRACT—The effects of various selective phosphodiesterase (PDE) inhibitors on muscle contractility in guinea pig ileal longitudinal smooth muscle were investigated. 1) 3-Isobutyl-1-methyl xanthine (IBMX) or zaprinast markedly inhibited the high K⁺- or carbachol (CCh)-induced contraction and increased cGMP content of the muscle strip in a concentration-dependent manner. However, these agents only slightly increased the cAMP content. Milrinone or Ro20-1724 also slightly inhibited the high K⁺- or CCh-induced contraction and increased the cAMP content, but did not increase cGMP. 2) In a fura2-loaded muscle, IBMX or zaprinast inhibited both contractions and the increase in intracellular Ca²⁺ ([Ca²⁺]ᵢ) level induced by high K⁺ or CCh, although the inhibitory effect on the [Ca²⁺]ᵢ level was smaller than that on muscle tension. 3) In α-toxin-permeabilized muscles, cGMP, IBMX or zaprinast significantly inhibited the Ca²⁺-induced contraction. These results suggest that IBMX and zaprinast inhibit muscle contraction in the ileal longitudinal smooth muscles mainly through an increase in cGMP and the inhibitory mechanism of IBMX or zaprinast is involved in the decreases in the [Ca²⁺]ᵢ level and sensitivity of contractile elements to Ca²⁺.

Keywords: Phosphodiesterase inhibitor, Ileal smooth muscle, Ca²⁺ sensitivity, Cyclic nucleotide, Cytosolic Ca²⁺

Adenosine 3',5'-cyclic monophosphate (cAMP) or guanosine 3',5'-cyclic monophosphate (cGMP) has been reported to play an important role in smooth muscle relaxation (1, 2). How smooth muscle contractility is affected by phosphodiesterase (PDE) inhibitors, which increase intracellular cAMP and/or cGMP, has been investigated (3, 4). Recently, PDE isozymes have been classified into types I to VII (5). Selective inhibitors have been developed for some of the subtypes (6): nicardipine (Ca²⁺ antagonist), W-7 and trifluoperazine (calmodulin inhibitor) and vinpocetine are specific for type I; milrinone, amrinone, vesnarinone, etc., for type III; rolipram and Ro20-1724 for type IV; zaprinast and dipyridamole for type V. These selective PDE isozyme inhibitors have been utilized as a vasodilator, antihypertensive drug and bronchodilator, and they have been employed to elucidate the mechanism of relaxation in smooth muscle. The effect of these inhibitors on the smooth muscle has been studied mainly in blood vessels and tracheae. The selective PDE III inhibitor milrinone markedly inhibited the serotonin-induced contraction in rat aorta (7) and the phenylephrine-induced contraction in guinea pig aorta (8). Other selective PDE III inhibitors also significantly inhibited serotonin-induced contractions (7) and norepinephrine-induced contractions (9–11) in rat aorta. On the other hand, selective PDE IV inhibitors caused relaxation in guinea pig trachea, and the combination of a PDE IV inhibitor with a PDE III inhibitor produced further relaxation (12). However, except for the study by Small et al. (13), there have been no other studies on the effects of selective PDE inhibitors on intestinal smooth muscle. Therefore, we investigated the mechanism of the inhibitory effect of the non-selective inhibitor 3-isobutyl-1-methyl xanthine (IBMX) and those of the selective inhibitors milrinone, Ro20-1724 and zaprinast on muscle contractility of guinea pig ileal longitudinal smooth muscle by measuring muscle tension, intracellular Ca²⁺ ([Ca²⁺]ᵢ) level, cAMP and cGMP contents in intact muscle strips and Ca²⁺-induced contractions in a muscle permeabilized by α-toxin or Triton X-100.

MATERIALS AND METHODS

Muscle preparations and tension measurement

Male guinea pigs (Hartley strain 300 – 400 g; Funabashi Farm, Funabashi) were bled after stunning, and then the
ileum of each animal was quickly removed. The longitudinal smooth muscle layer was stripped from the circular one as described by Paton and Aboo Zar (14). The muscle strips were about 5–8 mm in width and 15 mm in length. One end of each strip was bound to a glass holder and the other end was connected to a strain-gauge transducer (TB-611T; Nihon Kohden, Tokyo) with silk thread. The samples were equilibrated in a physiological salt solution (PSS) in an organ bath. The muscle tension was isometrically recorded.

**Assay of cAMP and cGMP contents in the ileal longitudinal muscle**

cAMP or cGMP content in the ileal longitudinal muscle was measured by enzyme immunoassay. After an incubation of the muscles with IBMX, milrinone, Ro20-1724 or zaprinast for 10 min in the presence of 1 nM carbachol (CCh), muscles were rapidly frozen in liquid nitrogen and stored at −80°C until homogenized in 6% trichloroacetic acid (0.4 ml). The homogenate was centrifuged at 3000 × g for 15 min, and the supernatant obtained was washed with water-saturated diethylether three times. cAMP or cGMP content was assayed by an enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, MI, USA). cAMP and cGMP contents are expressed as pmol/g wet wt.

**Simultaneous measurement of muscle tension and [Ca2+]i level**

The [Ca2+]i level was measured simultaneously with muscle tension as described previously (15). Muscle strips were incubated with 5 µM fura2/AM for 3–4 hr at room temperature. Then 0.02% cremophor EL, a noncytotoxic detergent, was added to increase the solubility of fura2/AM. One end of the muscle was pinned to the bottom of an organ bath filled with 8 ml of PSS, and the other end was attached to the transducer with silk thread. The muscle strip was kept horizontally in the organ bath. The muscle strip was alternately excited with light at 340 nm and 380 nm through the rotating filter wheel, and then the 500 nm emission was measured with a fluorimeter (CAF-100; Japan Spectroscopic Co., Ltd., Tokyo).

**Measurement of muscle tension in a permeabilized smooth muscle**

A thin longitudinal muscle strip, 5.0 mm in length and 0.2 mm in width, was prepared from the isolated ileum as described above. The muscle strip was held horizontally in an 1-ml organ bath. One end of each strip was fixed and the other end was connected to a strain-gauge transducer. The muscle tension was isometrically recorded.

**α-Toxin treated muscle:** A permeabilized muscle was made by treating the isolated muscle with a relaxing solution (RS-I) containing 40 µg/ml α-toxin for 20 min at room temperature as described by Nishimura and van Breemen (16) and Kitazawa et al. (17).

**Triton X-100 treated muscle:** The muscle was permeabilized as described by Sparrow et al. (18), with the following modification: The muscle strips were first incubated in solution A (pH 7.4) (see, Solutions) for 60 min at 4°C; then 1% Triton X-100 and 0.5 mM dithioerythritol (DTE) were added to this solution and incubation was carried out for 4 hr more at 4°C. After rinsing for 15 min in solution A containing 0.5 mM DTE, the strips were stored in solution B (pH 6.7) (see, Solutions) at −20°C for up to 5 days. The RS-II was entirely exchanged for each drug trial.

**Solutions**

PSS employed was a modified Tyrode’s solution (136.8 mM NaCl, 5.4 mM KCl, 1.5 mM CaCl2, 1.0 mM MgCl2, 11.9 mM NaHCO3 and 5.5 mM glucose), which was aerated with a 95% O2 and 5% CO2 at 37°C (pH 7.2). Hyperosmotically added 65.4 mM KCl (H-65K+) solution was made by increasing the concentration of KCl in the PSS.

In the experiment of the [Ca2+]i measurement, isosmotically added 77 mM KCl (I-77K+) solution was made by substituting an equimolar amount of K+ for Na+ in the PSS, because Himpens and Somlyo (19) suggested that a change of osmotic pressure induced a change of fura2-fluorescence.

In the experiment with the α-toxin permeabilized muscle, RS-I contained 130 mM potassium propionate, 4 mM MgCl2, 4 mM ATP, 20 mM tris-maleate, 2 mM creatine phosphate, 10 U/ml creatine phosphokinase and 2 mM ethyleneglycol bis-(β-aminoethylether)-N,N',N'-tetraacetic acid (EGTA). The solution was adjusted to pH 6.8 at 24°C with KOH (0.1 N). In the experiment with the Triton X-100 permeabilized muscle, solution A contained 20 mM imidazole, 5 mM EGTA, 50 mM KCl and 150 mM sucrose. Solution B contained 20 mM imidazole, 2 mM EGTA, 10 mM MgCl2, 1 mM NaN3, 0.5 mM DTE with 50% glycerol. RS-II contained 50 mM KCl, 20 mM imidazole, 8 mM MgCl2, 4 mM ATP, 3 mM KH2PO4, 3 mM K2HPO4, 0.1 µM calmodulin and 1 mM NaN3, adjusted to pH 6.7.

**Chemicals**

Chemicals used were IBMX, milrinone, zaprinast, CCh, verapamil, EGTA, cGMP, 8-bromoguanosine 3',5'-cyclic monophosphate (8-Br-cGMP, a membrane-permeant cGMP analogue), forskolin (Sigma Chemical Co., St. Louis, MO, USA); Ro20-1724 (4-(3-butoxy-4-methoxyphenyl)-methyl-2-imidazolidone) (LC Laboratories, New Boston, MA, USA); nitoroprusside (a soluble guanylate cyclase
activator) (Wako Pure Chemical, Osaka); fura2/AM (Dojindo Laboratories, Kumamoto); cremophor EL (Nacalai Tesque, Kyoto); and α-toxin (Research Biochemical International, Natick, MA, USA).

Statistics
Values are each expressed as the mean±S.E.M., and statistical analyses were performed by Student's t-test. Linear regression analysis was used to determine the relationship between relaxation and cGMP content.

RESULTS

Effects of PDE inhibitors on high K⁺- or CCh-induced contraction in ileal longitudinal muscle

When a contractile response induced by H-65K⁺ or 1 μM CCh reached a steady level about 15–20 min after an addition of the stimulant, each PDE inhibitor was added cumulatively. IBMX significantly inhibited H-65K⁺-induced contractions at 0.3 μM and CCh-induced contractions at 0.1 μM. Zaprinast also inhibited the H-65K⁺- or CCh-induced contraction in a concentration-dependent manner (Fig. 1). The inhibitions by these agents took as long as 10 min after their addition to reach steady state levels. Table 1 summarizes the concentrations of the PDE inhibitors producing 50% relaxation (IC₅₀) of H-65K⁺- or CCh-induced contraction. The results also showed that the inhibitory effects of milrinone and Ro20-1724 on the contractile response were remarkably smaller than those of IBMX and zaprinast.

Effects of PDE inhibitors on cGMP and cAMP contents

As shown in Fig. 2, both IBMX (3, 30 and 300 μM) and zaprinast (1, 10 and 100 μM) concentration-dependently increased the cGMP contents of the muscle strips in the presence of 1 μM CCh. The increase of cGMP content by 300 μM IBMX and 100 μM zaprinast reached 13.5 and 12.8 times above the control level, respectively. Milrinone

![Graphs showing the effects of PDE inhibitors on contraction and cGMP content](image)

Fig. 1. Effects of 3-isobutyl-1-methylxanthine (IBMX, A), milrinone (B), Ro20-1724 (C) and zaprinast (D) on the contraction induced by hyperosmotically added 65 mM KCl (H-65K⁺, □) or 1 μM carbachol (CCh, ▲). Preparations were precontracted with H-65K⁺ or CCh, and then the agents were added cumulatively. The maximum contractions induced by H-65K⁺ and 1 μM CCh in the absence of the agents were taken as 100%. **: Significant difference from each respective control with P<0.01. Each point represents the mean of 6–12 preparations. Vertical bars indicate the S.E.M.
Table 1. Effects of IBMX, milrinone, Ro20-1724 and zaprinast on the contraction induced by H-65K⁺ or CCh

<table>
<thead>
<tr>
<th>Agents</th>
<th>IC₅₀ (μM) H-65K⁺</th>
<th>IC₅₀ (μM) Carbachol (1 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBMX</td>
<td>66.9 ± 8.0 (10)</td>
<td>13.0 ± 1.3 (12)</td>
</tr>
<tr>
<td>Milrinone</td>
<td>&gt; 100 (10)</td>
<td>&gt; 100 (11)</td>
</tr>
<tr>
<td>Ro20-1724</td>
<td>&gt; 100 (8)</td>
<td>&gt; 100 (6)</td>
</tr>
<tr>
<td>Zaprinast</td>
<td>9.1 ± 0.8 (11)</td>
<td>8.1 ± 0.8 (12)</td>
</tr>
</tbody>
</table>

Values are each a mean ± S.E.M. Numbers in parentheses indicate the number of preparations used.

Fig. 2. Effects of IBMX (A) and zaprinast (B) on cGMP content in the ileal longitudinal muscle in the presence of 1 pM CCh. *: Significant difference from each respective control with P < 0.05, **: P < 0.01, respectively. Each point represents the mean of 4 experiments. Vertical bars indicate the S.E.M.

Fig. 3. Effect of IBMX (●), zaprinast (■) or nitroprusside (▲) on the relationship between the relaxation (ordinate) and the cGMP content (abscissa).

(100 μM) and Ro20-1724 (100 μM) did not significantly change the cGMP content (data not shown). Figure 3 illustrates the cGMP-relaxation relation obtained by the addition of IBMX, zaprinast or nitroprusside (1, 10 and 100 μM) in the presence of 1 μM CCh. It shows that despite a high correlation between inhibition of the muscle tension and the increase of cGMP content in the presence of IBMX, zaprinast or nitroprusside, the cGMP-relaxation relation of IBMX or zaprinast is located to the left of that obtained in the presence of nitroprusside. This indicates that a larger relaxation was induced in the presence of IBMX or zaprinast than in the presence of nitroprusside for a given increase in cGMP content.

In the presence of 1 μM CCh, both milrinone (1, 10 and 100 μM) and Ro20-1724 (1, 10 and 100 μM) increased the cAMP content of the muscle strips in a concentration-dependent manner (Fig. 4). Furthermore, IBMX (300 μM) or zaprinast (100 μM) also significantly increased the cAMP content of the muscle strips above the control, to levels that were similar to those induced by milrinone (100 μM) or Ro20-1724 (100 μM) (Fig. 5). Figure 6 illustrates the cAMP-relaxation relation obtained by the addition of milrinone, Ro20-1724 or forskolin (1, 10 and 100 μM) in the presence of 1 μM CCh. It shows that despite the high correlation between inhibition of the muscle tension and the increase of cAMP content in the presence of Ro20-1724 or forskolin, the cAMP-relaxation relation of Ro20-1724 is located to the left of that obtained in the presence of forskolin. This indicates that a larger relaxation was induced in the presence of Ro20-1724 than in the presence of forskolin for a given increase in cAMP content.
**Effects of IBMX, zaprinast, nitroprusside and 8-Br-cGMP on [Ca²⁺]ᵢ level**

The effects of IBMX, zaprinast, nitroprusside and 8-Br-cGMP on [Ca²⁺]ᵢ level were measured simultaneously with muscle tension by using a fluorescent Ca²⁺ indicator, fura2. I-77K⁺ or 1 μM CCh increased the muscle tension and the elevated [Ca²⁺]ᵢ level was indicated by a change in F340/F380. IBMX (300 μM) inhibited the I-77K⁺-induced contraction and [Ca²⁺]ᵢ level (Fig. 7, Table 2). The sequential addition of 10 μM verapamil further decreased both the muscle tension and [Ca²⁺]ᵢ level to the resting levels (Fig. 7). The inhibitory effects of IBMX on the CCh-induced contraction and elevated [Ca²⁺]ᵢ level were similar to those on the I-77K⁺-induced contraction (Fig. 7, Table 2). Zaprinast (100 μM) inhibited both the I-77K⁺ and 1 μM CCh-induced contractions and [Ca²⁺]ᵢ level (Fig. 8, Table 2). In summary, IBMX and zaprinast markedly inhibited the muscle tension but only slightly inhibited the [Ca²⁺]ᵢ level. Nitroprusside (100 μM) and 8-Br-cGMP (1 mM) also inhibited both the I-77K⁺- and CCh-induced contractions remarkably, but only slightly inhibited the [Ca²⁺]ᵢ level. The inhibition of the [Ca²⁺]ᵢ level by nitroprusside was smaller than that by IBMX (Table 2).
Effects of cGMP on Ca\(^{2+}\)-induced contractions in permeabilized muscle

As shown in Fig. 9, 1 \(\mu\)M Ca\(^{2+}\) induced a sustained contraction in the \(\alpha\)-toxin-permeabilized muscle. cGMP (100 \(\mu\)M) inhibited the 1 \(\mu\)M Ca\(^{2+}\)-induced contraction to 24.6\(\pm\)3.3\%. IBMX (300 \(\mu\)M) or zaprinast (100 \(\mu\)M) also inhibited the contraction to 49.9\(\pm\)3.4\% or 47.2\(\pm\)7.9\%, respectively.

In the Triton X-100-permeabilized muscle, 3 \(\mu\)M Ca\(^{2+}\) also induced a sustained contraction. IBMX (300 \(\mu\)M) or zaprinast (100 \(\mu\)M), however, rather enhanced the contractions to 11.6\(\pm\)2.6\% or 16.7\(\pm\)0.7\%, respectively.

DISCUSSION

The present study on the contractility of guinea pig ileal longitudinal muscle revealed that inhibitory effects of a non-selective PDE inhibitor, IBMX, and a selective PDE V inhibitor, zaprinast, were remarkable on high K\(^{+}\)- or CCh-induced contraction, while those of a selective PDE III inhibitor, milrinone, and a selective PDE IV inhibitor,
Ro20-1724, were small (Fig. 1, Table 1). It is well-known that selective PDE III or IV inhibitors induce a remarkable relaxation in vascular or tracheal smooth muscles. Therefore, these results suggest that the effect of selective PDE inhibitors on intestinal smooth muscles are different from that on vascular or tracheal smooth muscle.

IBMX and zaprinast increased the cGMP content of ileal longitudinal muscle in a concentration-dependent manner, although Ro20-1724 and milrinone had no effect on them. It is thought that increases in cGMP content by IBMX and zaprinast were caused by inhibition of PDE V. On the other hand, zaprinast also elevated the cAMP content of the muscle strips above the control level. There are two possibilities to explain this result: 1) Zaprinast slightly inhibits PDE isozymes (I, II, III, IV) that hydrolyze cAMP. 2) cGMP that was increased through an inhibition of PDE V by zaprinast inhibits PDE III that hydrolyzes cAMP (20).

In the present experiment, there was a high correlation between inhibition of the muscle tension and the increase of cGMP under the treatment with IBMX, zaprinast or sodium nitroprusside in the presence of CCh. Moreover, 8-Br-cGMP inhibited the high K+- or CCh-induced contraction in the ileal longitudinal muscle (Table 2). These results suggest that the relaxation induced by IBMX or zaprinast is involved in the increases of cGMP content. However, in comparison to the relaxation by nitroprusside, IBMX or zaprinast appears to induce a larger relaxation at a given cGMP content. It is assumed that the smaller relaxation by nitroprusside at a given cGMP content is involved in an overactivation of guanylate cyclase; if not, the greater relaxation by IBMX or zaprinast is influenced by another factor such as induction of cGMP sensitization in the intestinal smooth muscle.

It is known that isoproterenol that enhances cAMP contents of the muscle strip inhibits muscle tension in intestinal smooth muscles (21). In the present study, both milrinone and Ro20-1724 also concentration-dependently increased the cAMP content of the ileal longitudinal muscle. However, these agents had no effect on the cGMP content. There was a high correlation between the inhibition of muscle tension and the increases of cAMP under the treatment with Ro20-1724 or forskolin in the presence of CCh. However, compared to the relaxation by forskolin, Ro20-1724 appears to induce a larger relaxation at a given cAMP content. We think this difference can be explained by the same assumption for the difference among IBMX, zaprinast and nitroprusside with regards to the correlation between the cGMP content and relaxation. These results suggest that IBMX and zaprinast inhibit muscle tension mainly through the increase in cGMP, and Ro20-1724 inhibited it mainly through the increase in cAMP. Although milrinone increased the cAMP content,
it induced only a small relaxation. The data probably exclude the possibility that milrinone is involved in the cAMP sensitization in the smooth muscle.

In the present study, we investigated the relaxing mechanism of IBMX and zaprinast that show potent inhibition of muscle tension and the increase in cGMP content in ileal longitudinal muscle. It has been reported that sodium nitroprusside (22, 23), 8-Br-cGMP (22) and endothelium-derived relaxing factor (EDRF) (22) inhibited the norepinephrine-induced muscle tension through decreases in $[\text{Ca}^{2+}]$, level in rat aorta. In the present experiment, IBMX, zaprinast, nitroprusside and 8-Br-cGMP inhibited the high K$^+$- or CCh-induced contraction with a decrease in $[\text{Ca}^{2+}]$, level. From these results, 8-Br-cGMP and cGMP, which was increased by IBMX, zaprinast or nitroprusside, caused a decrease in the $[\text{Ca}^{2+}]$, level probably through the inhibition of Ca$^{2+}$ influx, the increase in Ca$^{2+}$ efflux and/or the increase in Ca$^{2+}$ sequestration in the ileal longitudinal muscle. However, the decrease of $[\text{Ca}^{2+}]$, level by cGMP seems to contribute partially to the inhibition of muscle tension because the decrease in $[\text{Ca}^{2+}]$, level was smaller than that in muscle tension. In the present experiment, cGMP, IBMX and zaprinast significantly inhibited the Ca$^{2+}$-induced contraction in the $\alpha$-toxin permeabilized ileal longitudinal muscle. Similarly, cGMP was reported to inhibit the Ca$^{2+}$-induced contraction in a concentration-dependent manner in rat mesenteric artery permeabilized by $\alpha$-toxin but retaining signal transduction systems (16). These results suggest that the inhibition of contraction by IBMX or zaprinast is partially related to the decrease in the sensitivity of contractile protein to Ca$^{2+}$. On the other hand, the possibility that the inhibition of Ca$^{2+}$-induced contraction by IBMX or zaprinast is directly related to an inhibition of contractile protein was excluded by the result that IBMX or zaprinast rather enhanced the Ca$^{2+}$-induced contraction in the Triton X-100 permeabilized muscle that lost its signal transduction system and ability of Ca$^{2+}$ sequestration in sarcoplasmic reticulum.

It is generally thought that a receptor agonist binds to a receptor and its stimulation generates inositol-1,4,5-trisphosphate and diacylglycerol by phosphatidyl-inositol turnover. In smooth muscles, these products are involved in contraction. Rapoport (24) reported that cGMP inhibited norepinephrine-induced contraction by inhibiting phosphatidyl-inositol turnover in the aorta. In the present experiment, zaprinast inhibited the high K$^+$- or CCh-induced contraction in ileal longitudinal muscle with a similar level of IC$_{50}$, suggesting that phosphatidyl-inositol turnover is not involved in the relaxation of ileal longitudinal muscle in the presence of high K$^+$ or CCh.

In summary, IBMX and zaprinast inhibit the muscle tension in guinea pig ileal longitudinal smooth muscle mainly through an increase in cGMP, while Ro20-1724 inhibits contraction mainly through an increase in cAMP. The inhibitory mechanism of IBMX or zaprinast is involved in the decrease in $[\text{Ca}^{2+}]$, level and the decrease in the sensitivity of contractile elements to Ca$^{2+}$.

Acknowledgments

We are grateful to Prof. K. Suzuki of the Division of Veterinary Physiology, Nippon Veterinary and Animal Science University for critical reading of the manuscript and thank Mr. M. Hirano and Mr. H. Yamamoto for assisting us with the experiments.

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