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Relaxant Effect of Spermidine on Acethylcholine and High K^+-induced Gastric Contractions of Guinea-Pig

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In our previous study, we found that spermine and putrescine inhibited spontaneous and acetylcholine (ACh)-induced contractions of guinea-pig stomach via inhibition of L-type voltage- dependent calcium current ($VDCC_L$). In this study, we also studied the effect of spermidine on mechanical contractions and calcium channel current (I_{Ba}), and then compared its effects to those by spermine and putrescine. Spermidine inhibited spontaneous contraction of the gastric smooth muscle in a concentration-dependent manner (IC_{50}=1.1±0.11 mM). Relationship between inhibition of contraction and calcium current by spermidine was studied using 50 mM high K^+-induced contraction: Spermidine (5 mM) significantly reduced high K^+ (50 mM)-induced contraction to 37±4.7% of the control ($p<0.05$), and inhibitory effect of spermidine on I_{Ba} was also observed at a wide range of test potential in current/voltage (*I/ V*) relationship. Pre- and post-application of spermidine (5 mM) also significantly inhibited carbachol (CCh) and ACh-induced initial and phasic contractions. Finally, caffeine (10 mM)-induced contraction which is activated by Ca^{2+}-induced Ca^{2+} release (CICR),` was also inhibited by pretreatment of spermidine (5 mM). These findings suggest that spermidine inhibits spontaneous and CCh-induced contraction via inhibition of $VDCC_L$ and Ca^{2+} releasing mechanism in guinea-pig stomach.

Key Words: Stomach, Relaxation, Calcium current, Ca^{2+} release, Spermidine

INTRODUCTION

Polyamines such as putrescine, spermidine, and spermine are natural compounds present in high concentrations (millimolar range) in eukaryotic and prokaryotic cells (Igarashi & Kashiwagi, 2000; Nishimura et al, 2006). In general, they are associated with pathologic functions and play an important role in cell growth and differentiation (Fernández et al, 1995). Polyamines are found in various types of muscles and affect membrane excitability and motility (Chideckel et al, 1985; Swärd et al, 1994; Tsvilovskyy et al, 2004). In the case of regulation of excitability, blocking effects of polyamines are known for a variety of channels, ranging from L-type voltage dependent Ca^{2+} channel ($VDCC_L$) to inwardly rectifying K^+ (IRK) channels (Kim, 2007; Lopatin & Nichols, 1996). Physiological effects of polyamines in gastrointestinal (GI) tract are known to inhibit gastric emptying by blocking $VDCC_L$ (thus inhibiting contractility) (Aihara et al, 1983; Gomez & Hellstrand, 1995; Tansy et al, 1982).

$VDCC_L$ have been described in GI smooth muscles (Katzka & Morad, 1989; Peulen et al, 2004), and known to play an essential role in the regulation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Kim et al, 1997). Because of these reasons, we studied the effect of spermine and putrescine on spontaneous and agonist-induced contraction of guinea-pig gastric smooth muslce. In guinea-pig stomach, putrescine and spermine inhibited spontaneous, high K^+- and acetylcholine (ACh)-induced contraction via inhibition of Ca^{2+} channel current (I_{Ba}). And much stronger inhibitory effect of spermine on those contractions than by putrescine was observed (Kim, 2007): For example, IC_{50} of spermine and putrescine for the inhibition of spontaneous contraction of gastric smooth muscle were 0.6 and 9.7 mM, respectively. In addition, 1 mM putrescine, spermidine and spermine inhibited peak amplitude of I_{Ba} to 46%, 51% and 81% of the control, respectively. Therefore, it seems that putrescine, spermine and spermidine might have an inhibitory effects on the regulation of gastric motility and I_{Ba} in guinea-pig stomach. Based on these backgrounds, we also studied the effect of spermidine on spontaneous, high K^+- and Ach (or carbachol, CCh)-induced contraction, including

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ABBREVIATIONS: Ach, Acethylcholine; $VDCC_L$, L-type voltage-dependent Ca^{2+} channel; I_{Ba}, Calcium channel current; CCh, Carbachol; CICR, Ca^{2+}-induced Ca^{2+} release; IRK, Inwardly rectifying K^+; GI tract, Gastrointestinal tract; EGTA, Ethyleneglycol bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid; SR, Sarcoplasmic reticulum; IICR, Inositol 1,4,5-triphosphate ($I_{ns}P_3$)-induced Ca^{2+}-release; RyR, Ryanodine-sensitive Ca^{2+} release.

I_{Ba} in guinea-pig stomach.

METHODS

Preparation of cells

Guinea-pigs of both gender, weighing 300~350 g, were anesthetized with fluoromethyl 2,2,2-trifluoro-1 (trifluoromethyl) ethyl ether (sevoflurane, Maruishi Pharma., Osaka, Japan), and exanguinated after stunning or decapitation. All experiments were performed in accordance with the guidelines for the animal care and use approved by the Chungbuk National University, The Physiological Society of Shanghai Jiaotong University. The antral portion of stomach was cut, and the mucosal layer was separated from the muscle layers in Ca^{2+}-free physiological salt solution (Ca^{2+}-free PSS). The circular muscle layer was dissected from the longitudinal layer using fine scissors and made into small segments (2×3 mm). These segments were incubated in Ca^{2+}-free PSS for 30 min at 4°C. Then, they were incubated for 15~25 min at 35°C in the digestion medium containing 0.1% collagenase (Wako, Japan), 0.05% dithiothreitol, 0.1% trypsin inhibitor and 0.2% bovine serum albumin. After the digestion, the supernatant was discarded, and the softened muscle segments were transferred into modified Kraft-Brühe (K-B) medium (Isenberg & Klöckner, 1982), and single cells were then dispersed by gentle agitation with a wide-bore glass pipette. Isolated gastric myocytes were kept in K-B medium at 4°C until use. All experiments were carried out within 8 hours of harvesting cells and performed at room temperature.

Whole-cell voltage clamp

Isolated cells were transferred to a small chamber on the stage of an inverted microscope (IMT-2, Olympus, Japan). The chamber was perfused with PSS (2~3 ml/min). Glass pipettes with a resistance of 2~5 MΩ were used to make a giga seal of 5~10 GΩ. Standard patch clamp techniques were used (Hamill et al, 1981). An axopatch-1C patch-clamp amplifier (Axon instruments, USA) was used to record membrane currents, and command pulses were applied by using IBM-compatible AT computer and pClamp software v.5.5.1. The data were displayed on a digital oscilloscope and a computer monitor.

Preparation of muscle strips

Vertical (25 ml) chambers were used for the mechanical experiment. For the measurement of mechanical contractions, muscle strips (5×10 mm) from the antral tissue with circular direction were prepared in isometric contractile measuring system. In this system, one end of tissue was tied tightly to fixed holder and the other side was also linked by hook type of holder to force transducer (Harvard, USA). The external solution was changed by solutions which had previously been incubated (bubbled with 5% CO_2/95% O_2, 36°C) in water bath before the application.

Solution and drugs

Ca^{2+}-PSS, containing (in mM): NaCl 135, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 1, glucose 10, and HEPES (*N*-[2-hydroxyethyl] piperazine-*N'*-[2-ethanesulphonic acid]) 10, was adjusted to pH 7.4 with NaOH. Modified K-B solution, containing (mM) L-glutamate 50, KCl 50, taurine 20, KH_2PO_4 20, $MgCl_2$ 3, glucose 10, HEPES 10, ethyleneglycol bis-(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) 0.5, was adjusted to pH 7.4 with KOH. Pipette solution, containing (mM) CsCl 110, TEA 20, EGTA 10, HEPES10, Na_2ATP 3, $MgCl_2$ 3.5, was adjusted to pH 7.3 with TRIZMA or CsOH. CO_2/bicarbonate-buffered Tyrode solution contained (in mM) NaCl 122, KCl 4.7, $MgCl_2$ 1, $CaCl_2$ 2, $NaHCO_3$ 15, KH_2PO_4 0.93, and glucose 11 (pH 7.3~7.4, bubbled with 5% CO_2/ 95% O_2). Equimolar concentration of Na^+ was replaced by K^+ for making high K^+ (50 mM) solution. All drugs used in this study were purchased from Sigma.

Statistics

Changes of relative contraction by polyamine were analyzed by measuring the amplitude of spontaneous contraction in the presence and absence of polyamines. In the case of high K^+ contraction, the amplitude of sustained contraction produced by 50 mM high K^+ in the presence of polyamines was compared to that of 50 mM high K^+. The data are expressed as means±SEM. Statistical significance was estimated by Student's *t*-test. $p<0.05$ was considered to be statistically significant.

RESULTS

Relaxation by spermidine in guinea-pig gastric smooth muscle

Effect of spermidine on isometric contraction of guinea-pig stomach was studied. As shown in Fig. 1A, spermidine inhibited spontaneous contraction of antral circular muscle in a concentration-dependent manner. The spontaneous contractions were reduced to 92±4.3, 88±4.6, 78±6.4, 65±5.3, 54±5.4, 40±4.5, 32±4.9, 19±2.3 and 12±3.3 % of the control at 0.02, 0.1, 0.2, 0.5, 1, 2, 3, 5, 10 and 20 mM of spermidine, respectively ($p<0.05$ at 0.1 mM~; n=13, 14, 14, 14, 13, 14, 13, 40, 12 and 4, respectively). IC_{50} of inhibition of contraction was 1.1±0.11 mM (Fig. 1A). To elucidate whether spermidine inhibited the contraction via inhibition of L-type Ca^{2+} current ($VDCC_L$), the effect of spermidine on 50 mM high K^+-induced contraction was also studied by spermidine pretreatment (Fig. 1B). Spermidine (5 mM) was applied for 15 minutes before application of high K^+ and its contractility was compared to that of the control. Spermidine reduced high K^+-induced contraction to 37±4.7% of the control ($p<0.05$; n=16). These findings indicate that spermidine-induced relaxation might be associated with the inhibition of $VDCC_L$.

Effects of spermidine on acethylcholine (ACh)- and carbachol (CCh)-induced contraction in guinea-pig stomach

ACh produces transient initial and tonic contraction, followed by a sustained phasic contraction (Sato K et al, 1994). Effect of spermidine on ACh (10 μM)- and CCh-induced initial and sustained phasic contraction was studied by pre- and post-application of spermidine. As shown in Fig. 2A, post-application of spermidine (5 and 10 mM) inhibited ACh-induced sustained phasic contraction to

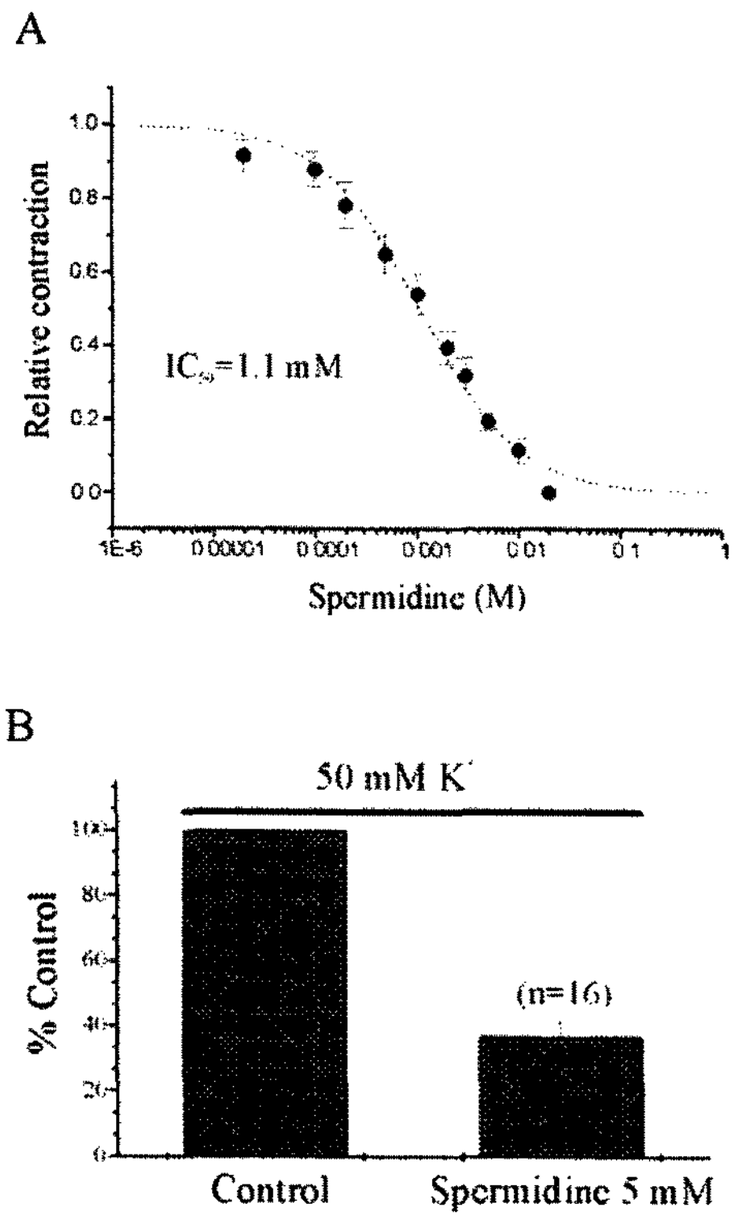


Fig. 1. Effect of spermidine on isometric and high K^+ (50 mM)-induced contraction in guinea-pig gastric smooth muscle. Effects of spermidine on isometric contraction were studied by using vertical chamber system. (A) Spermidine causes relaxation in a concentration-dependent manner. Relative contractions at various concentrations of spermine were plotted and fitted by the non-linear regression equation (IC_{50}=1.1 mM). (B) High K^+ (50 mM)-induced contraction was compared by pretreatment of spermidine (5 mM) before re-application of High K^+ solution. Such a high K^+ (50 mM)-induced contraction was significantly inhibited by the pretreatment with 5 mM spermidine ($p<0.05$).

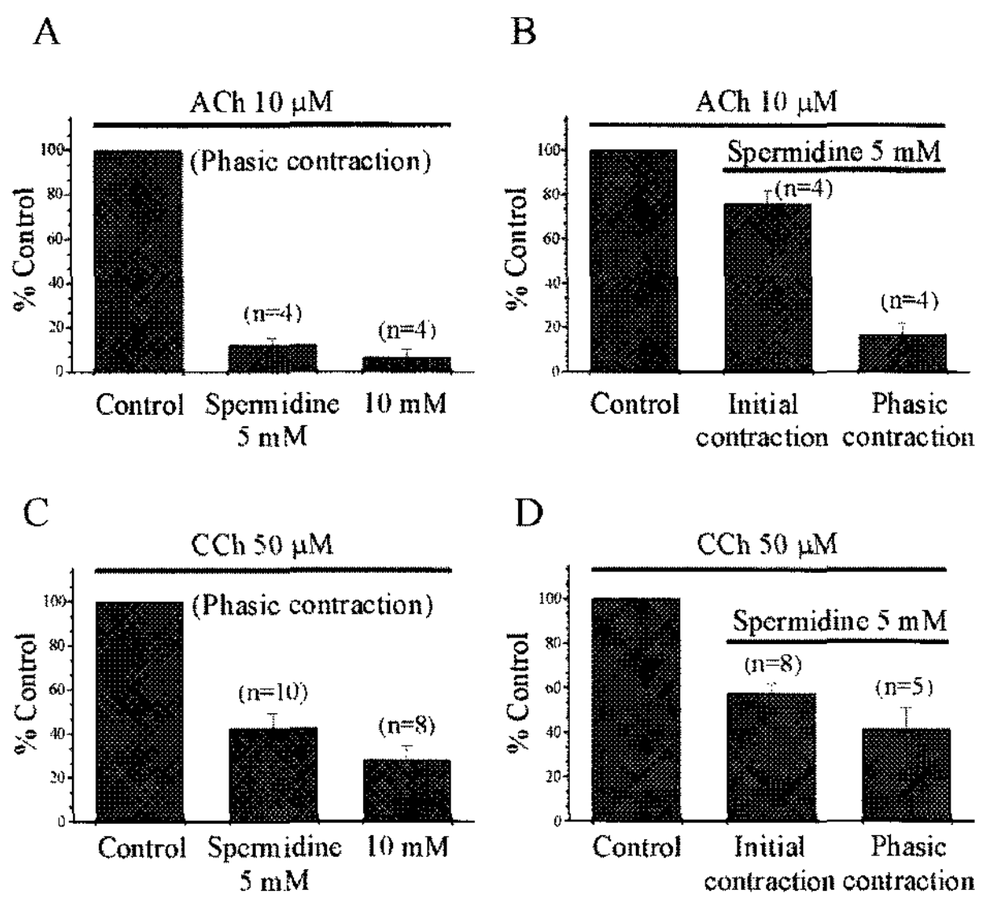


Fig. 2. Inhibitory effect of spermidine on muscaranic receptor agonist-stimulated contraction via regulation of $VDCC_L$. Acethylcholine (ACh, 10 μM) and carbachol (CCh, 50 μM) were applied to guinea-pig gastric smooth muscle. ACh- and CCh-induced transient initial and sustained phasic contractions were studied in the presence or absence of spermidine. (A and C) When 5 mM spermidine was applied to ACh- and CCh-induced sustained contractions, those contractions were inhibited to 12% and 43% of the control, respectively. (B) When 5 mM spermidine was pretreated before the second application of ACh, ACh-induced transient initial and sustained phasic contractions were inhibited to 76% and 17% of the control, respectively. (D) When 5 mM spermidine was applied before the second application of CCh, CCh-induced transient initial and sustained phasic contractions were also inhibited to 58% and 42% of the control, respectively.

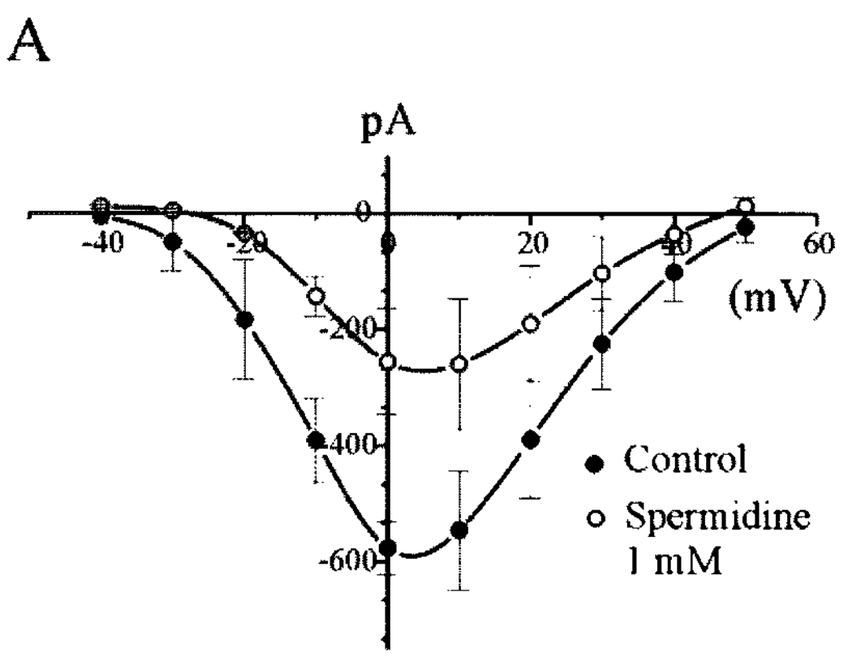
12±3.3 and 7±3.5% of the control, respectively ($p<0.05$; n=4). And pretreatment of spermidine (5 mM) before application of ACh (10 μM) also inhibited ACh-induced transient initial and sustained phasic contraction to 76±6.0% and 17±4.7% of the control, respectively ($p<0.05$; n=4; Fig. 2B). Spermidine also inhibited CCh (50 μM)-induced contractions. As shown in Fig. 2C, post-application of spermidine (5 and 10 mM) inhibited CCh-induced sustained phasic contraction to 43±6.7 and 28±6.7% of the control, respectively ($p<0.05$; n=10 and 8, respectively). And pretreatment of spermidine (5 mM) before application of CCh (50 μM) also inhibited CCh-induced transient initial and sustained phasic contraction to 58±4.4% and 42±9.5% of the control, respectively ($p<0.05$; n=8 and 5, respectively). Since ACh-induced sustained phasic contraction is sensitive to dihydropyridine such as nicardipine (Kim, 2007; Sato et al, 1994), these results indicate that polyamine-induced relaxation is associated with the inhibition of Ca^{2+} influx through $VDCC_L$ in guinea-pig stomach.

Effects of spermidine on the voltage activated calcium channel current (I_{Ba})

In our previous study, inhibitory effects of polyamines on I_{Ba} at 0 mV were compared with those of putrescine, spermidine and spermine (1 mM each). Spermidine decreased peak current of I_{Ba} at 0 mV to 51±5.7% of the control (Kim, 2007). In this study, current/voltage (I/V) relationship of I_{Ba} was also studied in the absence and presence of spermidine (Fig. 3). Extracellular Ca^{2+} was replaced by 10 mM Ba^{2+} after whole cell configuration was done. The membrane potential was held at −80 mV, and 10 mV step depolarization, ranging from −40 mV to 50 mV, was applied to the cell for 420 msec before and after the application of spermidine. Averaged responses in the presence and absence of spermidine (One mM) were plotted. As seen in Fig. 3A, 1 mM spermidine was found to decrease I_{Ba} at membrane potential range of −20 ~ +50 mV tested.

Effects of spermidine on caffeine-induced contraction

In addition to the effect of spermidine on muscrinic stimulated response by polyamines, we also studied the effect of spermidine on caffeine (10 mM)-induced contraction. Since caffeine releases Ca^{2+} from sarcoplasmic reticulum (SR), we tried to elucidate whether spermidine regulates caffeine-induced contraction via regulation of Ca^{2+}-induced Ca^{2+} release (CICR) mechanism (Iino M, 1990). As shown in Fig. 3B, caffeine-induced contraction was also significantly inhibited to 27±5.7% of the control by pretreatment of 5 mM spermidine ($p<0.05$; n=4). Since ACh (or



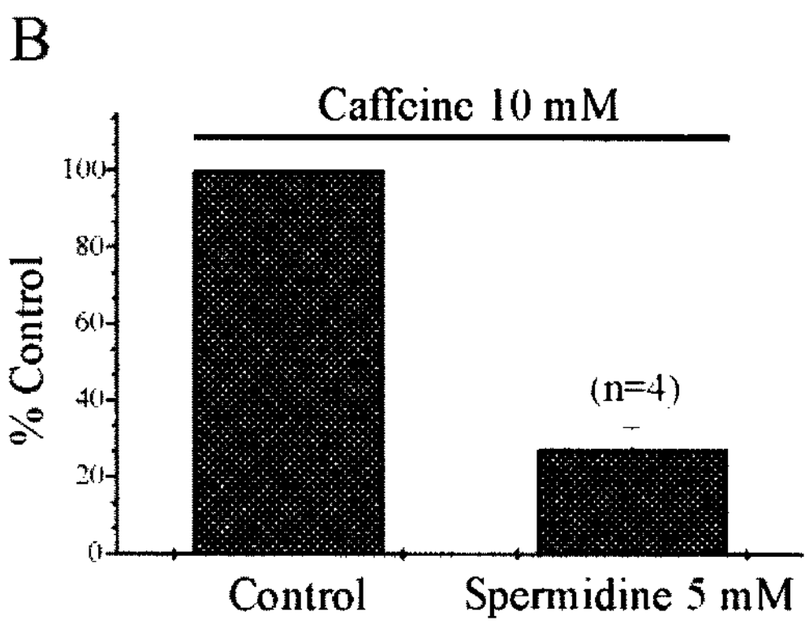


Fig. 3. Effect of spermidine on I_{Ba} and caffeine-induced contraction in guinea-pig gastric myocytes. (A) I_{Ba} was recorded under the condition in which extracellular Ca^{2+} was replaced by 10 mM Ba^{2+}. *I/V* relationship of I_{Ba} by spermidine is shown by averaged responses in the presence and absence of spermidine (closed circle, Control; open circle, 1 mM spermidine). Inhibition of I_{Ba} was observed at whole potential ranges tested. (B) Caffeine (10 mM)-induced contraction was compared with pretreatment of spermidine (5 mM) before re-application of caffeine. Caffeine-induced contraction was significantly inhibited by pretreatment with 5 mM spermidine ($p<0.05$).

CCh)-induced transient initial and caffeine-induced contractions were inhibited by spermidine, it might have an inhibitory effect on SR Ca^{2+} regulation, such as CICR mechanism, in gastric smooth muscle.

DISCUSSION

Relaxant effects of polyamines on smooth muscle contractility, including GI smooth muscle, have been reported (Gomez & Hellstrand, 1995; Hashimoto et al, 1973; Maruta et al, 1985; Kim, 2007). Recently, we also found inhibitory effect of spermine and putrescine on $VDCC_L$ with associated reduction of contractions (Kim, 2007). In this study, we examined whether the inhibitory potencies were different among polyamines. Spermine and putrescine inhibited spontaneous contractions with different potency (IC_{50}=0.6 mM and 9.7 mM, respectively).

As shown in Fig. 1A, the IC_{50} for inhibition of guinea-pig gastric smooth muscle by spermidine was 1.1 mM. Since spermine (2 mM) and putrescine (5 mM) suppressed 50 mM K^+-induced contraction to 16% and 53% of the control, respectively (Kim, 2007), and spermidine (5 mM) decreased the same contraction to 37% of the control (Fig. 1B), the potency of spermidine seems to be placed between spermine and putrescine. In fact, it was reported that putrescine has no effect on the contractility, whereas spermine (0.1~1 mM) showed potent blocking effect reversibly on taenia coli and intestine, and spermidine with weaker effect (Gomez & Hellstrand, 1999; Nilsson & Hellstrand, 1993). In single cell level, this potency order was also found in the inhibition of $VDCC_L$ in guinea-pig intestine (Gomez & Hellstrand, 1995). Unfortunately, we do not know why polyamines have different inhibitory potencies in smooth muscles (Gomez & Hellstrand, 1995; Gomez & Hellstrand, 1999; Kim YC, 2007; Nilsson & Hellstrand, 1993). Different binding affinity of polyamines on ion channels might possibly be responsible for this phenomenon (Schoemaker, 1992).

As shown in Fig. 1B, spermidine inhibited 50 mM K^+-induced contraction to 37% of the control, indicating that it decreases $VDCC_L$ in guinea-pig stomach too (Kim, 2007). In ion current level, its inhibitory effect on I_{Ba} was observed at whole potential range tested (Fig. 3A). Since spermidine inhibits $VDCC_L$ in guinea-pig stomach, we also studied the effect of spermidine on ACh- and CCh-induced sustained phasic contraction. In GI smooth muscle, ACh produces transient initial and tonic contraction, followed by a sustained phasic contraction (Sato et al, 1994). And $VDCC_L$ is known to be linked to ACh-induced sustained phasic contraction and ACh is also known to regulate Ca^{2+} current in GI smooth muscle (Kim, 2007; Sato et al, 1994). As shown in Fig. 2A and Fig. 2C, ACh- and CCh-induced sustained phasic contractions were significantly inhibited to 12±3.3% and 43±6.7% of the control, respectively, after application of spermidine (5 mM). Similarly, ACh- and CCh-induced sustained phasic contractions were also suppressed to 17±4.7% and 41±9.5% of the control by pre-treatment of spermidine (5 mM), respectively (Fig. 2B and Fig. 2D). Different levels of inhibitory activity on ACh- and CCh-induced phasic contraction by spermidine (Fig. 2) seem to be ascribed to the fact that CCh (50 μM) is stronger than ACh at same concentration. Because of this reason, inhibitory effect of spermidine on ACh-induced phasic contraction seems to be different from that of CCh. These data strongly imply that spermidine also has an inhibitory effect on gastric motility via inhibition of $VDCC_L$.

In our previous study, we already observed that spermine inhibited ACh-induced transient initial contraction by 53% (Kim, 2007). It is well known that $[Ca^{2+}]_i$ is one of important intracellular factors for maintenance of cellular functions, including the motility in smooth muscle (Kim, 1997). To date, two types of cytosolic Ca^{2+} stores have been identified as intracellular sources of Ca^{2+} mobilization in smooth muscles: The one is inositol 1,4,5-triphosphate ($InsP_3$)-induced Ca^{2+}-release (IICR) and the other is CICR (Iino, 1990). In smooth muscle, it has been established that stimulation with various agonists such as ACh releases Ca^{2+} from SR via IICR mechanism (Sato, 1994). As shown in Fig. 2B, 2D and 3B, spermidine also reduced ACh-, CCh- and caffeine-induced contraction by 24%, 42% and 73% of the control, respectively (n=4 each; Fig. 2B). In fact, ACh-induced initial transient contraction is responsible for IICR in GI smooth muscle (Sato, 1994), thus indicating that polyamines such as spermidine or spermine also inhibit smooth muscle contraction through inhibition of $[Ca^{2+}]_i$ releasing mechanism. In GI smooth muscle, transient

contractions caused by Ca^{2+} release in response to muscarinic stimulation was inhibited by spermine through inhibition of IP_3 production (Swärd et al, 1994). Therefore, the inhibition of IICR seemes to be underlying mechanism for inhibition of ACh-induced initial response in GI tract (Swärd et al, 1994; Tsvilovskyy et al, 2004).

In addition to modulation of IICR by polyamines, we also found regulation of CICR since caffeine-induced contraction was inhibited by spermidine (Fig 3B). CICR is a Ca^{2+} dependent gating of Ca^{2+} permeable channel (ryanodine-sensitive Ca^{2+} release, RyR) in SR membrane. Caffeine (10 mM), which opens RyR channels, is known to produce transient contraction (De Meis, 1967). In general, it shifts the Ca^{2+} sensitivity of RyR to lower concentrations and releases Ca^{2+} in intracellular space (Nagasaki & Kasai M, 1984). Experimentally, transient increase of $[Ca^{2+}]_i$ by extracellular application of caffeine has been regarded as an evidence for the presence of CICR in many kinds of cells (Kim et al., 1998). As shown in Fig. 3B, spermidine inhibited caffeine-induced contraction in guinea-pig stomach. Inhibitory effects of polyamine on caffeine-induced contraction via inhibition of SR Ca^{2+} channels unrelated to the effect on IP_3 production have also reported in GI and skeletal muscles (De Meis L, 1967; Palade, 1987). However, inhibitory effect could not be observed in other studies. Thus, there is a controversy on the direct inhibition of SR Ca^{2+} release mechanism by polyamines (Swärd et al, 1994). Therefore, further study on the regulation of $[Ca^{2+}]_i$ by polyamines is needed to verify underlying mechanism of this phenomenon.

Spermine ($NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$), spermidine ($NH_2(CH_2)_3NH(CH_2)_4NH_2$) and putrescine ($NH_2(CH_2)_4NH_2$) are aliphatic polyamines distributed widely in animal tissues (Wery, 1996). These compounds are synthesized inside of cell (Hougaard, 1987) and/or taken up from blood into cells after food intake in the gut (Nishimura, 2006; Wery, 1996). And intracellular concentration of spermidine (and/or spermine) in guinea-pig taenia coli and ileum ranges from between 0.1 to 0.2 mmol/L (Swärd et al, 1994; Nilsson & Hellstrand, 1993). Since they are synthesized in cell and taken up into cell, the site of their action is known to be mainly located at inside of cell (Hougaard et al, 1987). However, there is also a report to show that polyamines act on both sides of the membrane (Gomez & Hellstrand, 1999; Drouin & Hermann, 1994). In our previous study, we suggested that spermidine inhibits spontaneous and ACh-induced contraction via inhibition of $VDCC_L$ (Kim, 2007). It might be regulated either by intra- or extracellular (and/or both side of) spermidine in guinea- pig stomach since regulation of $VDCC_L$ on both sides of the membrane is already reported in GI smooth muscle cell (Gomez & Hellstrand, 1999; Drouin & Hermann, 1994).

Physiologically, polyamines are associated with many cellular functions such as protein synthesis and regulation of motility by ion channel regulation. Therefore, it is expected to play an important role in cellular homeostasis in GI tract (Nilsson & Hellstrand, 1993; Tabor & Tabor, 1984). In smooth muscles, polyamines inhibit smooth muscle contractions, as shown in our study (Kim, 2007): For example, spermine causes relaxation of respiratory smooth muscle (Chideckel et al, 1985), and also inhibits spontaneous contractions of uterine smooth muscle of rat (Maruta et al, 1985). Furthermore, polyamines have also been reported to inhibit gastric emptying, therefore, ulcer formation (Aihara, 1983; Martin et al, 1986). Because of these reasons, its inhibitory effect on gastric smooth muscle via inhibition of $VDCC_L$ and $[Ca^{2+}]_i$ releasing mechanisms may reflect physiological relevance.

In conclusion, spermidine inhibited spontaneous, muscarinic stimulated, and caffeine-induced contraction in guinea-pig stomach via inhibition of I_{Ba} and $[Ca^{2+}]_i$ releasing mechanisms.

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