Inhibitory mechanism of oscillation of carbachol-evoked inward cationic current by sodium nitroprusside in a single guinea pig ileal smooth muscle cell

Hong Ghi Lee

Department of Medical Science
The Graduate School, Yonsei University

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Directed by Professor Taick Sang Nam

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Hong Ghi Lee

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This certifies that the Doctoral Dissertation of Hong Ghi Lee is approved.

Thesis Supervisor: Taick Sang Nam

Jun Pyo Chung: Thesis Committee Member

Hye Yeon Lee: Thesis Committee Member

Seong Chun Kwon: Thesis Committee Member

Young Ho Lee: Thesis Committee Member

The Graduate School
Yonsei University

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TABLE OF CONTENTS

ABSTRACT ····· 1

I. INTRODUCTION ····· 3

II. MATERIALS AND METHODS ····· 4

1. Isolation of the longitudinal smooth muscle layer from the guinea pig ileum ····· 4

2. Preparation of cells ····· 5

3. Whole-cell voltage clamp ····· 5

4. Permeabilized preparation ····· 6

5. Solutions ····· 7

6. Chemicals ····· 8

7. Statistics ····· 8

III. RESULTS ····· 9

1. CCh-evoked inward cationic current (I_{cat}) oscillations ····· 9

2. Properties of the CCh-evoked inward cationic current oscillations ····· 9

A. Effects of 10 mM BAPTA and 4.6 mM Ca^{2+} in the pipette solution on I_{cat} oscillations ····· 9

B. Effect of thapsigargin on I_{cat} oscillations ····· 10

C. Effect of heparin on I_{cat} oscillations ····· 11

D. Effect of ruthenium red on I_{cat} oscillations ····· 12

3. Characteristics of inhibitory effect of sodium nitroprusside (SNP), NO donor, on I_{cat} oscillations ····· 12

A. Effect of SNP on I_{cat} oscillations ····· 12

B. Effect of Rp-8-Br-cGMP, Rp-diastereometer of cGMP, on the inhibition of I_{cat} oscillations by SNP ····· 12

C. Effect of SNP on I_{cat} when $[Ca^{2+}]_i$ is held to resting level ····· 13

D. Effects of SNP on the caffeine and $InsP_3$-induced tension developments in permeabilized muscle ····· 14

IV. DISCUSSION ····· 17

V. CONCLUSION ····· 21

REFERENCES ····· 22

ABSTRACT (IN KOREAN) ····· 24

LIST OF FIGURES

Fig. 1. Carbachol (CCh) (1㎛M)-induced inward cationic current oscillations (I_{cat} oscillations) and effects of sodium nitroprusside (SNP) on I_{cat} oscillations at holding potential (H.P.) of -60 mV in guinea pig ileal smooth muscle cells ····· 9

Fig. 2. Effect of BAPTA (10 mM) and Ca^{2+} (4.6 mM) in patch pipette solution on I_{cat} oscillations and effect of SNP on sustained current evoked by CCh (1㎛M) ····· 10

Fig. 3. Effect of thapsigargin on I_{cat} oscillations ····· 10

Fig. 4. Effect of heparin and ruthenium red on I_{cat} oscillations ····· 11

Fig. 5. Effect of ODQ and 8-Br-cGMP on the inhibitory effect of SNP on I_{cat} oscillations ····· 13

Fig. 6. Effect of Rp-8-Br-cGMP on the inhibitory effect of SNP on I_{cat} oscillations ····· 14

Fig. 7. Effect of SNP on the tension responses to application of caffeine in α-toxin-permeabilized longitudinal smooth muscle of the guinea-pig ileum ····· 15

Fig. 8. Effect of SNP on the tension responses to application of D-myo-$InsP_3$ in α-toxin-permeabilized longitudinal smooth muscle of the guinea-pig ileum ····· 16

Fig. 9. Comparison of the effect of SNP on the tension responses to caffeine and D-myo-$InsP_3$ in α-toxin-permeabilized longitudinal smooth muscle of the guinea-pig ileum ····· 17

Abstract

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To investigate sodium nitroprusside (SNP) effect on I_{cat} oscillations in guinea pig ileal longitudinal smooth muscle cells, the effect of SNP on I_{cat} oscillations was investigated using the patch-clamp technique. Futhermore, to gain more insight about the mechanism of this, the effect of SNP on Ca^{2+}-release from the intracellular store by caffeine and $InsP_3$, respectively, was tested using permeabilized preparation of longitudinal smooth muscle strip. SNP (10㎛M) completely inhibited I_{cat} oscillations evoked by 1㎛M carbachol (CCh). This effect was partially reversible. ODQ (1㎛M), a soluble guanylate cyclase inhibitor, alone showed no effect on I_{cat} oscillations, but it almost completely prevented inhibition of I_{cat} oscillations by SNP. In contrast, a membrane permeable analogue of cGMP, 8-Br-cGMP (30㎛M), in patch pipette solution completely abolished I_{cat} oscillations. A recently synthesized membrane-permeable Rp-8-Br-cGMP which is a highly specific antagonist of the activation of cGMP-dependent kinase by cGMP almost completely abolished the inhibitory SNP effect on I_{cat} oscillations. To avoid the effect of $[Ca^{2+}]_i$ on I_{cat}, $[Ca^{2+}]_i$ in ileal smooth muscle cells was held to the resting level using 10 mM BAPTA and 4.6 mM Ca^{2+} in pipette solution ($[Ca^{2+}]_i$= ~100 nM). In this case, CCh evoked only sustained component of I_{cat} without any oscillations and SNP did not decrease the amplitude of sustained component of I_{cat}. SNP significantly inhibited Ca^{2+} release evoked by $InsP_3$, not by caffeine in the permeabilized preparations of guinea pig longitudinal smooth muscle.

In conclusion, it has been demonstrated that the inhibitory effects of SNP on I_{cat}

oscillations are mediated, in part, by inhibition of $InsP_3$ receptor, neither by inhibition of CCh-evoked inward cationic channels nor by muscarinic receptors in the plasma membrane. This inhibition seems to be mediated by an increased cGMP concentration, in a phosphorylation dependent (i.e. PKG-dependent) fashion.

Key words: gastrointestinal smooth muscle, carbachol-evoked cationic current, sodium nitroprusside, oscillation

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I. INTRODUCTION

Many different kinds of cells exhibit oscillating changes in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) which usually occur in response to hormones and neurotransmitters, and sometimes spontaneously. Because of the unique occurrence of $[Ca^{2+}]_i$ oscillations and the existence of cellular functions which are mediated by an increase in $[Ca^{2+}]_i$, possible roles for the $[Ca^{2+}]_i$ oscillations have been suggested.[1]

Oscillations of $[Ca^{2+}]_i$ evoked by acetylcholine (ACh) or carbachol (CCh) were observed in a single intestinal smooth muscle cell.[2,3] They are associated with ACh or CCh-evoked inward cationic current oscillations (I_{cat} oscillations).[4] There is evidence that the activation of a G-protein results in opening of cationic channels and that this is further potentiated by increases in $[Ca^{2+}]_i$. During the CCh stimulation, Ca^{2+} inhibition of $InsP_3$-induced Ca^{2+} release at some critical level of $[Ca^{2+}]_i$ allows Ca^{2+} stores to refill and leads to a fall in $[Ca^{2+}]_i$ to the level at which $InsP_3$ can release Ca^{2+} from stores again.[5] Therefore, the oscillations of $[Ca^{2+}]_i$ arising from muscarinic stimulation is closely correlated with I_{cat} oscillations and periodic release of Ca^{2+} from the internal stores by $InsP_3$ formed through phosphatidyl-inositol breakdown induces I_{cat} oscillations.[6] Although

physiological role of CCh-evoked inward cationic current (I_{cat}) oscillations has not been elucidated, these results suggest that I_{cat} oscillations may play a role in modulating intestinal contractility.

Nitric oxide (NO) and compounds capable of liberating NO relax various types of smooth muscle,[7,8] and are known to activate soluble guanylate cyclase with a subsequent increase in cyclic guanosine monophosphate (cGMP) levels,[9] which in turn causes activation of G kinase.[10] This cascade results in a reduction of $[Ca^{2+}]_i$ through a series of complex and poorly understood mechanisms.[11] In the gastrointestinal tract, Kwon et al.[12] reported that sodium nitroprusside (SNP) inhibits smooth muscle contraction stimulated by CCh mainly by decreasing $[Ca^{2+}]_i$ which resulted from the combination of the inhibition of voltage-dependent Ca^{2+} channels, the inhibition of I_{cat} and the activation of Ca^{2+}-activated K^+ currents. So there is a possibility that decreased $[Ca^{2+}]_i$ by SNP affects the I_{cat} oscillations which is closely correlated with $[Ca^{2+}]_i$ oscillations.

Taken together, it is possible that I_{cat} oscillations play a role in producing and maintaining the intestinal contractility in physiological conditions, and NO, inhibitory neurotransmitter, regulates intestinal contractility by modulating I_{cat} oscillations, but there has been no report investigating NO effect on I_{cat} oscillations in the gastrointestinal tract.

To investigate these possibilities, we used the patch-clamp technique to record I_{cat} oscillations and tested SNP effect on I_{cat} oscillations. Futhermore, to gain more insight about the mechanism of the effect of SNP on I_{cat} oscillations, SNP effect on Ca^{2+}-release from the intracellular store by caffeine and $InsP_3$, respectively, was tested using permeabilized preparation of longitudinal smooth muscle strip.

II. MATERIALS AND METHODS

1. Isolation of the longitudinal smooth muscle layer from the guinea pig ileum

Guinea-pigs of either sex, weighing 300～350 g, were exsanguinated after stunning. The ileum was isolated and cut into segments of 3 to 4 cm in length and placed in the physiological salt solution (PSS) (composition given below). The longitudinal muscle layer of ileal segments was

peeled from the circular layer and washed in PSS.

2. Preparation of cells

The longitudinal muscle layer of the ileum was cut into small pieces and placed in Ca^{2+}-free PSS. The Ca^{2+}-free PSS was then replaced with PSS containing 30μM Ca^{2+} (low Ca^{2+} PSS), and 30-minute incubations at 37°C were carried out in the fresh low Ca^{2+} PSS containing collagenase (0.3 mg/ml), papain (0.6 mg/ml), and bovine serum albumin (1 mg/ml). After this enzyme digestion, tissue fragments were suspended in a fresh 120μM Ca^{2+} containing PSS and gently agitated. The resulting suspension was centrifuged at 600 xg for 2 minutes, and the cells were resuspended in a 0.5 mM Ca^{2+} containing PSS, aliquoted into 12 mm cover glasses and stored in a humidified atmosphere at 4°C. Experiments were carried out within 12 hours of harvesting (22～24°C).

3. Whole-cell voltage clamp

Whole-cell membrane currents were recorded at room temperature using standard patch-clamp techniques. The patch pipette had a resistance of 3～6 MΩ when filled with a pipette solution. Membrane currents were measured with an Axoclamp 200 A voltage-clamp amplifier (Axon Instrument, Foster City, CA, USA). Command pulses were applied using an IBM-compatible computer and pCLAMP (version 6.0) software. The data were filtered at 5 kHz and displayed on an oscilloscope, a computer monitor, and a pen recorder.

CCh was used at 1μM concentration because CCh 1μM was close to the concentrations of neurotransmitters in the enteric nervous system (about 0.1～1μM) and an effective dose inducing I_{cat} oscillations.[3,13] In the experiment required to hold $[Ca^{2+}]_i$ to resting level, 10 mM BAPTA (buffering $[Ca^{2+}]$ to an almost constant level) and 4.6 mM Ca^{2+} were included in the pipette solution, and their effects on I_{cat} oscillations in response to CCh 1μM were monitored. In another experiment, thapsigargin was applied to the bathing solution during I_{cat} oscillations to inhibit Ca^{2+}-transporting ATPase in the Ca^{2+} store membrane.[14,15] In the third experiment, heparin was added

in the pipette solution at a concentration of 5 mg/ml to inhibit $InsP_3$ receptor, and similarly, ruthenium red was used at a concentration of 10μM to block ryanodine receptors on the sarcoplasmic reticulum.

SNP (10μM), NO donor, was added to the bathing solution to study its effect on I_{cat} oscillations. A single ileal smooth muscle cell was pretreated with ODQ, a soluble guanylate cyclase inhibitor, at a concentration of 1μM and 8-Br-cGMP, a membrane permeable analogue of cGMP, at a concentration of 30μM for 3～5 minutes to study their effects on the inhibitory effect of SNP on I_{cat} oscillations. Also the cell was dialysed intracellularly with Rp-8-Br-cGMP, a highly specific antagonist of the activation of cGMP-dependent kinase by cGMP, at a concentration of 30μM for 3 minutes to study its effect on the inhibitory effect of SNP on I_{cat} oscillations.

4. Permeabilized preparation

A small muscle strip, 4～6 mm in length and 0.2～0.3 mm in width, was prepared from the longitudinal muscle layer of the ileum, was mounted horizontally in a 1 ml organ chamber and had one end fixed to the chamber and the other attached to an isometric force transducer. The organ chamber was filled with a PSS kept at 23°C and the muscle strip was equilibrated under a tension of 150～180 mg for 30～60 minutes. Then, permeabilization of the muscle cells was performed by incubating the muscle strip with *Staphylococcus aureus* α-toxin (10μg protein/ml) in a Ca^{2+}-containing solution (pCa 6) for approximately 30～60 minutes until the gradual rise in tension reached a steady level. After permeabilization, the muscle strip was bathed in the relaxing solution containing 2 mM EGTA. (R_I solution, composition given below).

In control experiments, intracellular Ca^{2+} stores of the permeabilized ileal muscle were loaded with Ca^{2+} by replacing the bath medium (R_I solution) with Ca^{2+}-containing solution (pCa 6) for 10 minutes. Then, a relaxing solution (R_I solution) was reintroduced for 5 minutes. Caffeine or $InsP_3$ was applied for 1～1.5 minutes by replacing the R_I solution with another relaxing solution (R_{II} solution, composition given below) to which these drugs were added (first experiment). After

that, permeabilized muscle strip were equilibrated for 20 minutes before restarting the same experiment once more (second experiment). Since the Ca^{2+}-releasing effect mediated by G-protein-coupled receptors is usually unstable in chemically skinned smooth muscle, and GTP is a substrate of guanylate cyclase for cGMP synthesis, GTP (100μM) was allowed to be present during the application of caffeine or $InsP_3$ and introduction of R_I solution (time for SNP pretreatment) in order to replenish the loss of endogenous GTP.[16,17]

The data were given in the following form:

$$ratio\ (\%) = \frac{A_{drug\text{-}2}}{A_{drug\text{-}1}} \times 100$$

where $A_{drug\text{-}1}$ and $A_{drug\text{-}2}$ are the tension amplitudes induced by drugs in the first and second experiments, respectively.

In experiments which investigate the effect of SNP on Ca^{2+} release from store by these drugs, the protocols were the same as the control experiment except for the addition of SNP in relaxing solution during reintroduction of R_I solution and the applications of these drugs in the second experiments.

5. Solutions

The PSS used for cell isolation and the bathing solution for CCh-evoked inward cationic current (I_{cat}) recording had the following composition (mM): 126 NaCl, 6 KCl, 2 $CaCl_2$, 1.2 $MgCl_2$, 14 glucose, 10.5 N-[2-hydroxyethyl]piperazine-N-[2-ethansulphonic acid] (HEPES) (titrated to pH 7.4 with NaOH).

Ca^{2+}-free PSS was prepared by simply omitting $CaCl_2$ from the PSS composition. The patch pipette solution for oscillatory I_{CCh} recording had the following composition (mM): 134 CsCl, 1.2 $MgCl_2$, 4 MgATP, 0.3 Na_2GTP, 0.05 ethylene glycol-bis(β-aminoethylether)-N,N,N'N'-tetraacetic acid (EGTA), 10 phosphocreatine, 10 glucose and 10 HEPES (titrated to pH 7.2 with CsOH). In some experiments, for $[Ca^{2+}]_i$ to be clamped close to a resting value typical for intestinal smooth

muscle to minimize the influence of changes in $[Ca^{2+}]_i$ on I_{cat}, a mixture of 10 mM 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) and 4.6 mM Ca^{2+} were used instead of 0.05 mM EGTA because BAPTA is superior to EGTA in buffering $[Ca^{2+}]_i$ to an almost constant level (calculated $[Ca^{2+}]_i$ = 100 nM). The relaxing solution for permeabilized preparation had the following composition (mM): 130 K propionate, 4 $MgCl_2$, 5 Na_2ATP, 2 creatine phosphate, 10 creatine phosphokinase, 20 Tris-maleate, 2 EGTA (for R_I solution) or 0.05 EGTA (for R_{II} solution) (pH 6.8). Added to the relaxing solution were the mitochondrial inhibitor, carbonyl cyanide p-trifluoromethoxy phenylhydrazone (1㎛M), and the protease inhibitor, E-64 (1㎛g/ml). Ca^{2+} concentrations were changed by adding an appropriate amount of $CaCl_2$. The apparent binding constant of EGTA for Ca^{2+} was considered to be 1 M at pH 6.8 and 20°C.

6. Chemicals

Sodium nitroprusside (SNP), ethylene glycol-bis(β-aminoethylether)-N,N,N'N'- tetraacetic acid (EGTA), carbachol (CCh), caffeine, guanosine triphosphate sodium salt (Na_2GTP), adenosine triphosphate magnesium salt (MgATP), N-[2-hydroxyethyl]piperazine-N-[2-ethansulphonic acid] (HEPES), 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA), 8-bromo-guanosine 3',5'-cyclic monophosphate (8-Br-cGMP), Rp-8-bromo-cyclic guanosine 3',5'-cyclic monophosphate (Rp-8-Br-cGMP), creatine phosphokinase, nifedipine, heparin, *Staphylococcus aureus* α-toxin, E-64, D-myo-inositol-1,4,5-trisphosphate, 1H-(1,2,4)oxadiazole[4,3-*a*]quinoxaline-1-one (ODQ) were purchased from Sigma (St Louis, MO, USA). All other chemicals were of the highest grade among the commercially available products.

7. Statistics

All results are expressed as mean±S.E.. Statistical significance of differences between given sets of data was evaluated by Student's *t* test for single comparison. A *P* value of <0.05 was considered significant.

III. RESULTS

1. CCh-evoked inward cationic current (I_{cat}) oscillations

Oscillatory inward cationic current was evoked by 1μM CCh applied to the bathing solution of cells voltage-clamped at -60 mV at least 3 minutes after break-through.

In most cells held at -60 mV, CCh produced oscillatory I_{cat} responses (Fig. 1). The I_{cat} oscillations were evoked usually from a slightly sustained I_{cat} component with a more or less regular frequency or they occurred without the development of noticeable sustained current, and they persisted for the entire or early period during the application of CCh (2～10 min). The mean of oscillation frequency among cells held at -60 mV was 0.17±0.02 Hz (n=32).

2. Properties of the CCh-evoked inward cationic current oscillations

A. Effects of 10 mM BAPTA and 4.6 mM Ca^{2+} in the pipette on I_{cat} oscillations

Ten mM BAPTA and 4.6 mM Ca^{2+} in the pipette solution completely prevented the cells from generating inward oscillatory currents in response to CCh at 1μM concentration, and if any inward current was evoked, it was a sustained one (Fig. 2) (n=8).

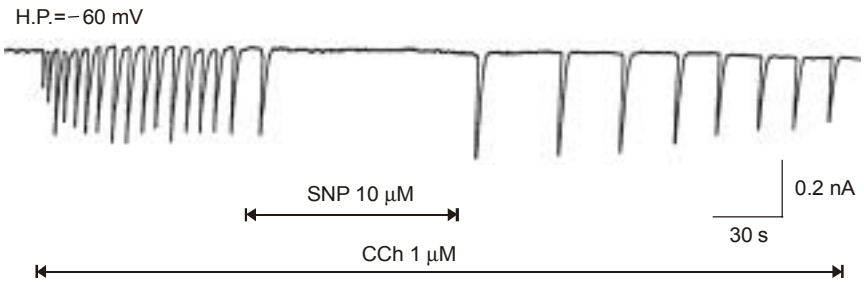

Fig. 1. Carbachol (CCh) (1μM)-induced inward cationic current oscillations (I_{cat} oscillations) and effects of SNP on I_{cat} oscillations at holding potential (H.P.) of -60 mV in guinea pig ileal smooth muscle cells. I_{cat} oscillations were completely inhibited by 10μM sodium nitroprusside (SNP).

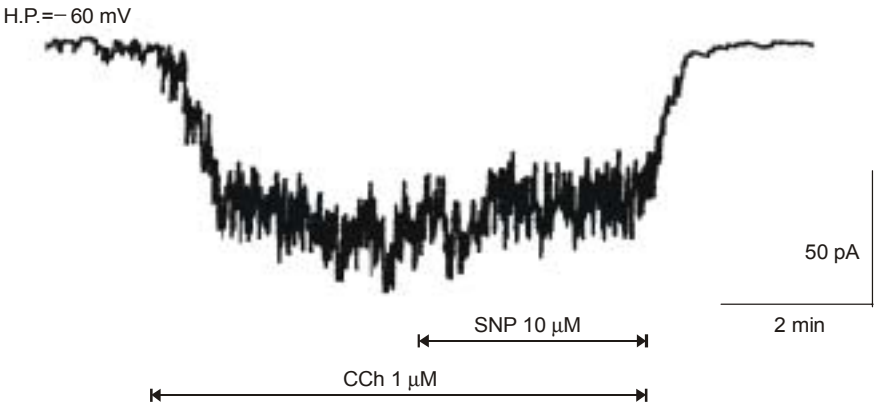

Fig. 2. Effect of BAPTA (10 mM) and Ca^{2+} (4.6 mM) in patch pipette solution on I_{cat} oscillations and effect of SNP on sustained current evoked by CCh (1μM). In the presence of 10 mM BAPTA and 4.6 mM Ca^{2+}, I_{cat} oscillations were disappeared remaining only small-sustained current. SNP did not show any inhibitory effect on the sustained current.

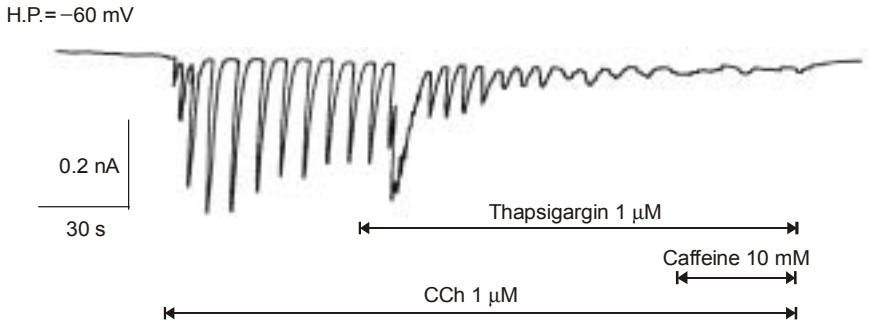

Fig. 3. Effect of thapsigargin on I_{cat} oscillations. Thapsigargin (1μM) applied in the continued presence of CCh gradually and completely inhibited I_{cat} oscillations. When 10 mM caffeine was applied subsequent to the disappearance of the oscillations, it produced no increase in the sustained I_{cat} component.

B. Effect of thapsigargin on I_{cat} oscillations

To investigate the role of the internal Ca^{2+} store on I_{cat} oscillations, the effect of thapsigargin, a potent inhibitor of Ca^{2+}-transporting ATPase, was tested. As shown in Fig. 3, I_{cat} oscillations were inhibited by thapsigargin (1μM) and its effect occurred with a latency of up to 1 minute during which the oscillations decreased in amplitude gradually (n=5). When 10 mM caffeine was applied subsequent to the disappearance of the oscillations, it produced no increase in the sustained

I_{cat} component (Fig. 3).

C. Effect of heparin on I_{cat} oscillations

To investigate the role of $InsP_3$ sensitive Ca^{2+} pool on I_{cat} oscillations, the effect of heparin, inhibitor of $InsP_3$ receptors in Ca^{2+} stores, was tested. The application of heparin at a concentration

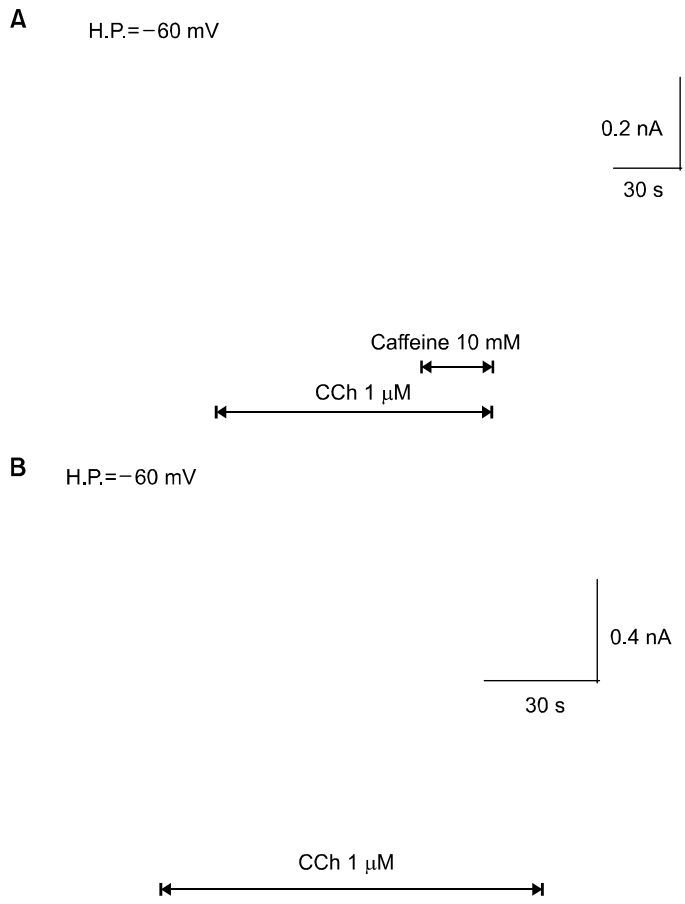

Fig. 4. Effect of heparin and ruthenium red on I_{cat} oscillations. (A) Current record from the cell dialysed intracellularly with heparin (5 mg/ml) for about 3 min. CCh (1μM) evoked only a small, slowly developing, sustained inward current without any oscillatory current. Caffeine (10 mM) was applied in the presence of CCh. (B) Current record from the cell with pretreatment of ruthenium red (10μM). CCh 1μM was applied after 5 min pretreatment. No substantial difference was observed compared with normal cell (see Fig. 1).

of 5 mg/ml for 5～10 minutes prevented the generation of oscillatory inward currents in response to CCh except initial large inward current (Fig. 4A). After that, when caffeine (10 mM) was applied in the presence of intracellular heparin, a brief, large inward current was evoked (n=4).

D. Effect of ruthenium red on I_{cat} oscillations

To investigate the role of ryanodine sensitive Ca^{2+} pool on I_{cat} oscillations, the effect of ruthenium red, inhibitor of ryanodine receptors in Ca^{2+} stores, was tested. In all of five cells pre-treated with ruthenium red (10μM) for at least 5 minutes, CCh could still evoke oscillatory I_{cat} (n=4) (Fig. 4B).

3. Characteristics of inhibitory effect of SNP, NO donor, on I_{cat} oscillations

A. Effect of SNP on I_{cat} oscillations

To investigate the role of NO on I_{cat} oscillations, the effect of SNP, NO donor, was tested. Fig. 1 demonstrates a typical example of the inhibitory effect of SNP (10μM) on I_{cat} oscillations in guinea pig ileal smooth muscle cells. SNP (10μM) completely inhibited I_{cat} oscillations in all cells tested (n=6). This effect was reversible but the frequency of oscillations was somewhat decreased when bathing solution was replaced with PSS without SNP.

To investigate the role of guanylate cyclase for the SNP effect on I_{cat} oscillations, ODQ, a soluble guanylate cyclase inhibitor, was tested. As shown in Fig. 5A, ODQ (1μM) alone showed no effect on I_{cat} oscillations, but it almost completely prevented inhibition of I_{cat} oscillations by SNP (n=4) (Fig. 5A). In contrast, 8-Br-cGMP, a membrane permeable analogue of cGMP, (30 μM) in patch pipette solution completely abolished I_{cat} oscillations (n=6) (Fig. 5B).

B. Effect of Rp-8-Br-cGMP, Rp-diastereometer of cGMP, on the inhibition of I_{cat} oscillations by SNP

To investigate the role of cGMP-dependent protein kinase for the SNP effect on I_{cat} oscillations, Rp-8-Br-cGMP, a highly specific antagonist of the activation of cGMP-dependent kinase by cGMP, was tested. As shown in Fig. 6, Rp-8-Br-cGMP almost completely abolished the inhibitory SNP effect on I_{cat} oscillations (n=4).

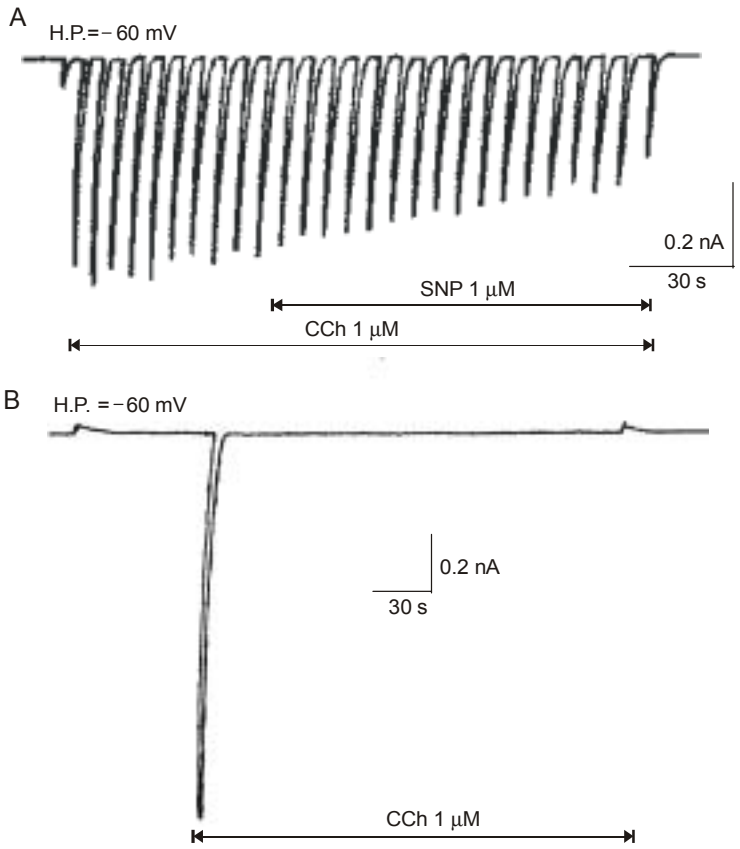

Fig. 5. Effect of ODQ and 8-Br-cGMP on the inhibitory effect of SNP on I_{cat} oscillations. (A) Current record from the cell with pretreatment of ODQ (1μM). CCh (1μM) applied after 5 min pretreatment still evoked I_{cat} oscillations. When SNP was applied in the presence of ODQ, inhibitory effect of SNP on I_{cat} oscillations was nearly completely prevented. (B) Current record from the cell dialysed intracellularly with 8-Br-cGMP (30μM) for about 3 min. CCh (1μM) did not evoke any oscillatory current in the presence of 8-Br-cGMP in pipette solution.

C. Effect of SNP on I_{cat} when $[Ca^{2+}]_i$ is held to resting level

As shown above, 10 mM BAPTA and 4.6 mM Ca^{2+} included in the pipette solution prevented I_{cat} oscillatory current remaining only small-sustained current. SNP (10μM) failed to decrease the amplitude of sustained current of I_{cat} (Fig. 2).

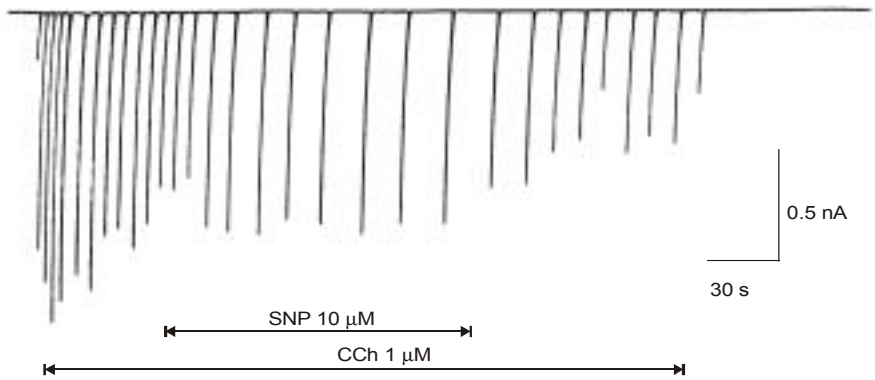

Fig. 6. Effect of Rp-8-Br-cGMP on the inhibitory effect of SNP on I_{cat} oscillations. Current record from the cell dialysed intracellularly with Rp-8-Br-cGMP (30μM) for about 3 min. When SNP (10μM) was applied in the presence of Rp-8-Br-cGMP in pipette solution, the inhibitory effect of SNP on I_{cat} oscillations was nearly completely prevented.

D. Effects of SNP on the caffeine or $InsP_3$-induced tension developments in permeabilized muscle

In the control experiment with caffeine, tension arose and reached a peak within 3 minutes during Ca^{2+} loading (see methods). The peak tension remained almost unchanged or declined gradually by less than 30%. Caffeine (10 mM), applied 5 minutes after reintroduction of relaxing solution (R_I solution) following Ca^{2+}-loading, produced a transient rise in tension due to the release of stored Ca^{2+}. The tension responses to caffeine reached a peak within 1 minute, and then declined close to their levels before caffeine application in its continued presence. The second application of caffeine evoked a tension with the amplitude very similar to that of the first application (Fig. 7A) (96.8±2.4%, n=4). Similarly, in SNP experiments, the amplitudes of tension in the second application of caffeine in the presence of SNP were not significantly attenuated compared to those of the first applications in the absence of SNP (Fig. 7B) (94.1±3.0%, n=4). Namely, SNP did not significantly inhibit Ca^{2+} release evoked by caffeine.

D-myo-$InsP_3$ (30μM) applied in the same way as used for caffeine and elicited a transient rise in tension (Fig. 8A). The tension responses to $InsP_3$ were generally smaller and slower in declining

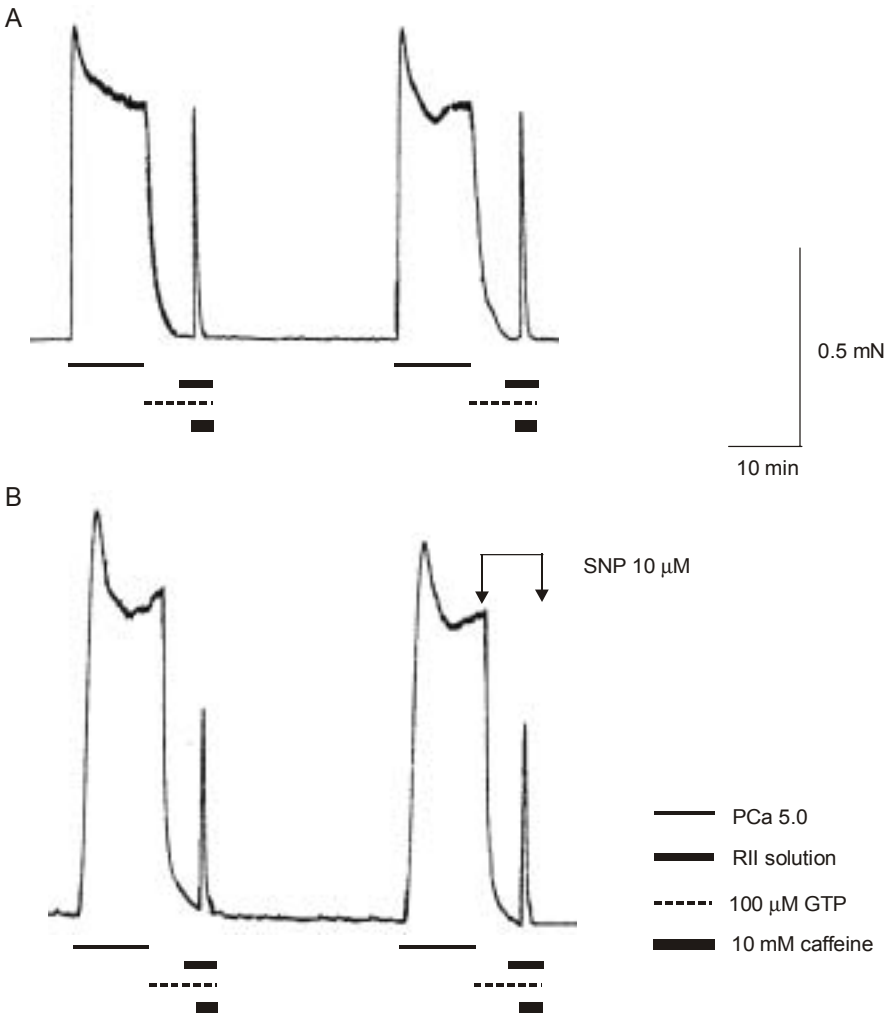

Fig. 7. Effect of SNP on the tension responses to application of caffeine in α-toxin-permeabilized longitudinal smooth muscle of the guinea-pig ileum. (A) The record of tension response to caffeine in control experiment. The tension responses to caffeine reached a peak within 1 min, and then declined close to their levels before caffeine application in its continued presence. The second application of caffeine evoked tension with amplitude very similar to that of the first application (n=4). (B) The record of SNP effect on the tension response to caffeine in control experiment. The amplitudes of tension in the second application of caffeine in the presence of SNP were not significantly attenuated compared to those of the first applications in the absence of SNP (n=4).

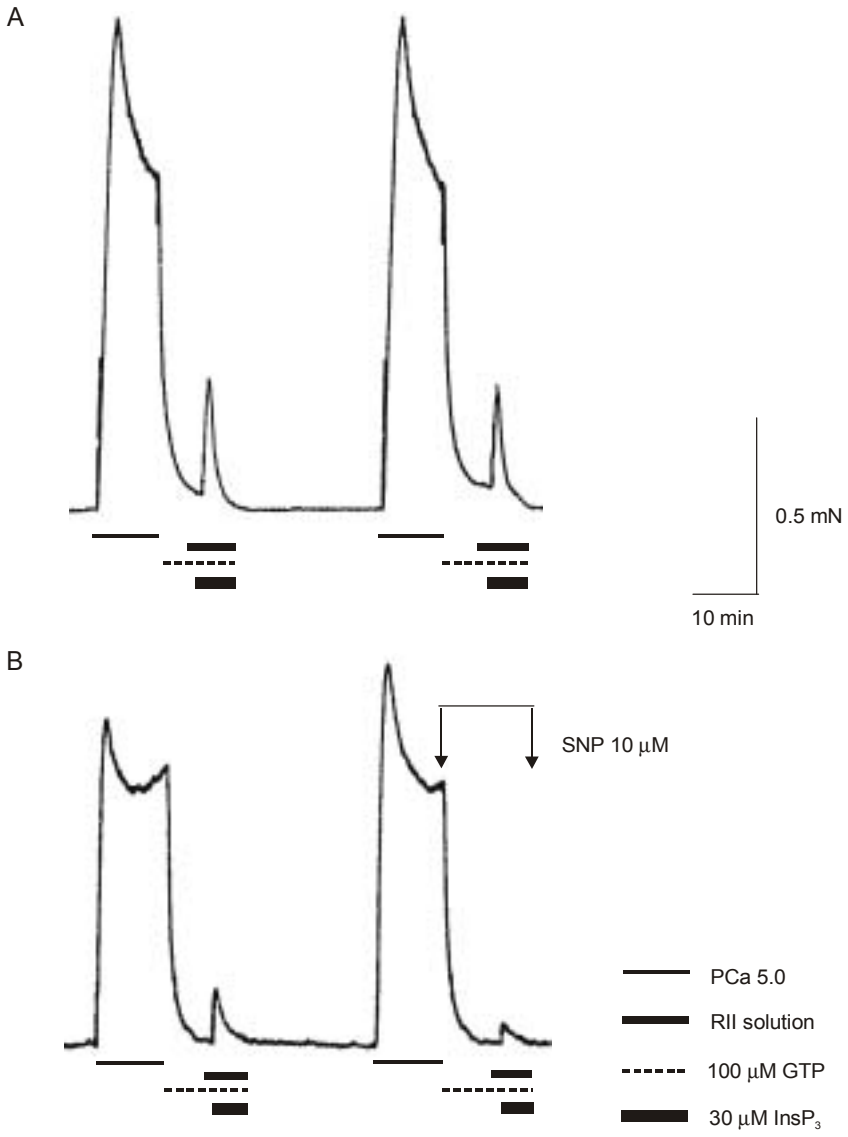

Fig. 8. Effect of SNP on the tension responses to application of D-myo-$InsP_3$ in α-toxin-permeabilized longitudinal smooth muscle of the guinea-pig ileum. (A) The record of tension response to D-myo-$InsP_3$ (30μM) in control experiment. The second application of $InsP_3$ evoked a tension with amplitude very similar to that of the first application (n=4). (B) The record of SNP effect on tension response to D-myo-$InsP_3$ in control experiment. The amplitudes of tension in the second application of $InsP_3$ in the presence of SNP were significantly attenuated compared to those of the first applications in the absence of SNP (n=5).

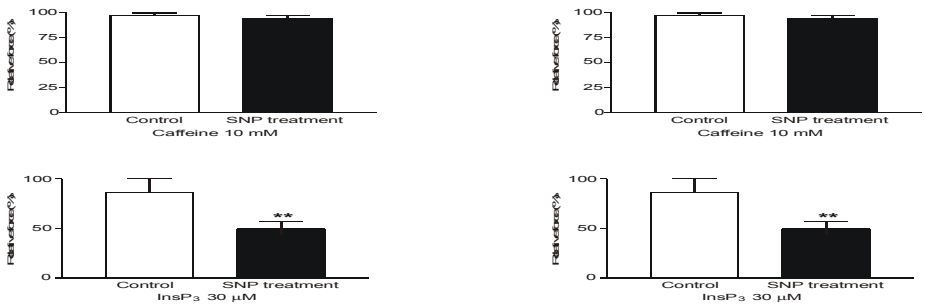

Fig. 9. Comparison of the effect of SNP on the tension responses to caffeine and D-myo-$InsP_3$ in β-toxin-permeabilized longitudinal smooth muscle of the guinea-pig ileum. The data are shown as mean± S.E.M.. $^{**}P$ value < 0.05.

from the peak, compared with those to 10 mM caffeine. The second application of $InsP_3$ evoked a tension with the amplitude very similar to that of the first application (Fig. 8A) (93.3±6.67%, n=4). In SNP experiments, the amplitudes of tension in the second application of $InsP_3$ were significantly attenuated compared to those of the first applications (Fig. 8B) (48.9±7.8%, n=5). These data are summarized in Fig. 9.

IV. DISCUSSION

The main findings of this study are as follows: (1) in guinea pig ileal longitudinal smooth muscle cells, CCh evokes I_{cat} oscillations *via* $InsP_3$-induced Ca^{2+} release, not *via* Ca^{2+}-induced Ca^{2+} release, as previously reported,[3] (2) SNP, NO donor, potently inhibits I_{cat} oscillations *via* cGMP dependent and G-kinase dependent manners, (3) the inhibitory effect of SNP on I_{cat} oscillations may be mediated, in part, by alteration of the IP_3 receptor (channel), neither by inhibition of muscarinic receptor nor by CCh-evoked inward cationic channels, (4) the inhibitory effect of SNP on CCh-induced Ca^{2+} release may be mediated by alterations in the $InsP_3$ receptor (channel).

To investigate whether the properties of I_{cat} oscillations are the same as those of previously described or not, the following experiments with the same protocol that had been previously reported[3] were carried out on a single smooth muscle cell of the guinea pig ileum. In summary,

CCh evoked an oscillatory inward cationic current. I_{cat} oscillations were abolished by inclusion of 10 mM BAPTA and 4.6 mM Ca^{2+} in the pipette solution. Thapsigargin, a potent inhibitor of Ca^{2+}-transporting ATPase, made I_{cat} oscillations disappear. Heparin (inhibitor of $InsP_3$ receptors in Ca^{2+} stores) blocked the generation of current oscillations, but ruthenium red (inhibitor of ryanodine receptors in Ca^{2+} stores) had no effect on I_{cat} oscillations. The results obtained in the above experiments suggested that I_{cat} oscillations is steeply dependent on $[Ca^{2+}]_i$, and Ca^{2+}-induced Ca^{2+} release (CICR) *via* the ryanodine receptor had a minor, if any, role in I_{cat} oscillations but $InsP_3$-induced Ca^{2+} release (IICR) *via* $InsP_3$ receptor played an essential role in sustaining I_{cat} oscillations.

A substantial part of the neuromuscular regulation of the enteric nervous system is the nonadrenergic noncholinergic (NANC) inhibitory neurotransmission of the gastrointestinal smooth muscle. There is a strong evidence indicating that NO and a related NO donating substance are the major candidate transmitters of NANC innervation in the gastrointestinal tract.[18-20] Because NO is an unstable gaseous agent, NO donors such as glyceryl trinitrate, SNP, and 3-morpholino-sydnonimine (SIN-1) have been used to study the effects of NO. In the present study, SNP (10 μM) completely inhibited I_{cat} oscillations in all cells stimulated by 1μM CCh (Fig. 1), and ODQ (1μM), a soluble guanylate cyclase inhibitor, almost completely prevented inhibition of I_{cat} oscillations by SNP (Fig. 5A). A membrane permeable analogue of cGMP, 8-Br-cGMP, (30μM) in patch pipette solution completely abolished I_{cat} oscillations (Fig. 5B). These results suggest that SNP exerted the inhibitory actions on I_{cat} oscillations *via* an increased production of cGMP. It has generally been accepted that the cellular effects of cGMP are mediated by a protein kinase G (PKG) which phosphorylates a variety of functional proteins including ion channels and thereby alters their function.[21] Therefore, it is possible that SNP inhibit I_{cat} oscillations mediated by a PKG. In the present study, a recently synthesized membrane-permeable Rp-8-Br-cGMP, a highly specific antagonist of the activation of cGMP-dependent kinase by cGMP, almost completely abolished the inhibitory SNP effect on I_{cat} oscillations (Fig. 6). These results suggest that SNP exerts the

inhibitory actions on I_{cat} oscillations *via* activation of PKG by increased cGMP, which is consistent with the data previously reported.[21]

In general, inhibitory or relaxing action of NO donors or cGMP-increasing agents on smooth muscle is thought to be mediated by altered properties of various cellular proteins which participate in Ca^{2+} homeostasis as well as those directly associated with contractile event.[7,8] As mentioned above, NO is known to activate soluble guanylate cyclase with a subsequent increase in cGMP level. Increased cGMP triggers a reduction in $[Ca^{2+}]_i$ through the activation of PKG. Activated PKG phosphorylates several key target proteins all involved in the control of $[Ca^{2+}]_i$. The effects of cGMP on these targets include, at least, 1) activation of Ca^{2+}-activated K^+ channels,[22,23] 2) inhibition of membrane Ca^{2+} channels,[12,24] 3) inhibition of the $InsP_3$ receptor,[25,26] 4) inhibition of $InsP_3$ generation.[27] Phosphorylation of these target proteins reduces $[Ca^{2+}]_i$ and results in relaxation of smooth muscle. In the present study, SNP (10㎛M) inhibited I_{cat} oscillations in a single smooth muscle cell voltage-clamped at -60 mV in which almost voltage-dependent Ca^{2+} channels are deactivated and 130 mM Cs in the pipette solution totally block voltage-dependent, Ca^{2+}-dependent K^+ channels. Thus, it is unlikely that the inhibitory effect of SNP on I_{cat} oscillations is mediated by the inhibition of voltage-dependent Ca^{2+} current or the activation of Ca^{2+}-activated K^+ current. Considering all these circumstances, at least two possibilities can be suggested about the mechanism of the inhibitory SNP effect on I_{cat} oscillations: 1) inhibition of CCh-evoked inward cationic channel and their accessory proteins or muscarinic receptors in the plasma membrane, 2) reduction of $[Ca^{2+}]_i$ by inhibiting IICR from the intracellular Ca^{2+} stores. The first possibility is not likely because SNP did not inhibit the amplitude of sustained component of I_{cat} when $[Ca^{2+}]_i$ in ileal smooth muscle cell was held to the resting level using 10 mM BAPTA and 4.6 mM Ca^{2+} in pipette solution ($[Ca^{2+}]_i$= ~100 nM). As for the second possibility, it is previously reported that after its generation by phospholipase C (PLC) activity, $InsP_3$ acts as a second intracellular messenger for smooth muscle contraction by binding to a specific receptor. The $InsP_3$ receptor is a channel protein located in the sarcoplasmic reticulum, which opens when bound to $InsP_3$. The opening $InsP_3$ receptor

channel permits Ca^{2+} efflux into the cytoplasm, resulting in contraction of the smooth muscle.[25] As mentioned above, the $InsP_3$ receptor is one of the best-known substrates of PKG. Phosphorylation of the $InsP_3$ receptor reduces the channel activity in response to $InsP_3$, leading to a decrease in $[Ca^{2+}]_i$ and smooth muscle relaxation.[25,26] In the present study, CCh evokes I_{cat} oscillations via IICR, not via CICR in the intestinal smooth muscle. Consequently, inhibition of $InsP_3$-induced Ca^{2+} release results in the inhibition of I_{cat} oscillations (Fig. 4A). So it is possible that SNP inhibit I_{cat} oscillations by inhibiting $InsP_3$ receptor (channel). To gain more exact insight into this possibility, the effects of SNP on the caffeine or $InsP_3$-induced tension developments were tested in *Staphylococcus aureus* α-toxin-permeabilized longitudinal smooth muscle of the guinea-pig ileum. As shown in Fig. 8, SNP inhibited Ca^{2+} release evoked by $InsP_3$, not by caffeine, in *Staphylococcus aureus* α-toxin permeabilized preparation. Caffeine is known to release stored Ca^{2+} *via* ryanodine receptor (channel) without stimulating $InsP_3$ production. This result was not inconsistent with data previously reported.[3,5] Therefore, the inhibitory effects of SNP on I_{cat} oscillations are mediated, in part, by inhibition of IICR through alterations of essential properties of the $InsP_3$ receptor such as reduced $InsP_3$ affinity and decreased Ca^{2+}-mobilizing efficacy, not by inhibition of CICR. Meanwhile, it has been shown that in bovine aortic smooth muscle, cGMP inhibited the vasopressin- or GTPγS-induced activation of PLC, and the hydrolysis of ATP was a prerequisite (i.e. PKG mediated), which is consistent with our observations (also see below). This effect is likely to occur at the level of the G protein or the interaction of activated G protein with PLC, since inhibition was not observed when PLC was directly activated by Ca^{2+}.[28] It is also possible that inhibition of I_{cat} oscillations by SNP may involve reduced $InsP_3$ production. Unfortunately, this possibility was not tested in this study. A further study that investigates the effect of SNP on $InsP_3$ production evoked by CCh using the radioisotope assay is warranted. Taken together, the results obtained above suggest that the inhibitory effects of SNP on CCh-induced Ca^{2+} release and I_{cat} oscillations are mediated by alterations in the $InsP_3$ receptor, neither by inhibition of CCh-evoked inward cationic channels and their accessory proteins nor by muscarinic

receptors in the plasma membrane; however, the possibility that the inhibitory SNP effect on I_{cat} oscillations can be mediated by reducing $InsP_3$ production should be tested in the future.

V. CONCLUSION

To investigate SNP effect on I_{cat} oscillations in guinea pig ileal longitudinal smooth muscle cells, the effect of SNP on I_{cat} oscillations was investigated using the patch-clamp technique to record I_{cat} oscillations. Furthermore, to gain more insight about the mechanism of this, the effect of SNP on Ca^{2+}-release from the intracellular store by caffeine and $InsP_3$, respectively, was tested using permeabilized preparation of longitudinal smooth muscle strip. The results of the experiments were as follows:

1. SNP (10μM) completely inhibited I_{cat} oscillations evoked by 1μM CCh. This effect was partially reversible.

2. ODQ (1μM), a soluble guanylate cyclase inhibitor, alone showed no effect on I_{cat} oscillations, but it almost completely prevented inhibition of I_{cat} oscillations by NO. In contrast, a membrane permeable analogue of cGMP, 8-Br-cGMP, (30μM) in patch pipette solution completely abolished I_{cat} oscillations.

3. A recently synthesized membrane-permeable Rp-8-Br-cGMP which is a highly specific antagonist of the activation of cGMP-dependent kinase by cGMP almost completely abolished the inhibitory SNP effect on I_{cat} oscillations.

4. To avoid the effect of $[Ca^{2+}]_i$ on I_{cat}, $[Ca^{2+}]_i$ in ileal smooth muscle cells was held to the resting level using 10 mM BAPTA and 4.6 mM Ca^{2+} in pipette solution ($[Ca^{2+}]_i$= ~100 nM). In this case, CCh evoked only sustained component of I_{cat} without any oscillations and SNP did not decrease the amplitude of sustained component of I_{cat}.

5. SNP significantly inhibited Ca^{2+} release evoked by $InsP_3$, not by caffeine in permeabilized preparations of the guinea pig longitudinal smooth muscle.

In conclusion, it has been demonstrated that the inhibitory effects of SNP on I_{cat} oscillations

are mediated, in part, by inhibition of $InsP_3$ receptor, not by inhibition of CCh-evoked inward cationic channels or muscarinic receptors in the plasma membrane, and this inhibition seems to be mediated by an increased cGMP concentration, in a phosphorylation dependent (i.e. PKG-dependent) fashion. These results provide a more comprehensive mechanism for the inhibitory action of NO in regulating the intestinal motility and may suggest a vital role for NO to prevent or treat intestinal motility disorder caused by cholinergic overstimulation.

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국문요약

기니 픽 회장 평활근에서 carbachol에 의한 내향성 양이온 전류 oscillation에 대한 sodium nitroprusside의 억제 기전

＜지도교수 **남 택 상**＞

연세대학교 대학원 의학과

이 홍 기

기니 픽 회장 종주근에서 carbachol (CCh)에 의한 내향성 양이온 전류의 oscillation (I_{cat} oscillations)에 대하여 NO 공여물질인 sodium nitroprusside (SNP)의 효과를 막전압 고정법을 사용하여 조사하였고, 나아가 구체적인 SNP 억제 효과기전을 알기 위해서 종주근 조직을 permeabilization시켜서 caffeine과 $InsP_3$에 의한 세포 내 Ca^{2+} 저장소에서 Ca^{2+} 유리에 대하여 SNP가 어떤 효과를 가지고 있는지 관찰하였다.

SNP (10㎛)은 CCh에 의한 내향성 양이온 전류 oscillation (I_{cat} oscillations)을 완전히 그리고 가역적으로 억제하였다. 이 I_{cat} oscillations에 대한 SNP의 억제효과는 1㎛ ODQ의 전처치에 의하여 거의 완전히 차단되었다. 반면에 막투과성이 있는 cGMP analogue인 8-Br-cGMP (30㎛)를 patch pipette 용액에 투여한 결과 I_{cat} oscillations이 완전히 소실되었다. cGMP-dependent protein kinase 의 선택적 길항제인 Rp-8-Br-cGMP (30 ㎛)를 patch pipette 용액에 투여한 후에는 SNP의 억제효과가 현저하게 차단되었다. 세포 내 Ca^{2+} ($[Ca^{2+}]_i$)의 I_{cat}에 대한 영향을 배제하기 위하여 세포 내 calcium을 10 mM BAPTA와 4.6 mM Ca^{2+}을 사용하여 약 100 nM로 고정한 경우에 CCh은 oscillations이 전혀 없는 sustained inward current를 발생시켰다. 이러한 실험 조건에서 SNP는 sustained inward current의 크기에 아무런 영향을 주지 못했다. 막투과도를 증가시키는 실험에서 SNP는 $InsP_3$에 의한 세포 내 저장소로부터의 Ca^{2+} 유리는 현저히 억제하였지만 caffeine에 의한 유리는 억제하지 못하였다.

위의 결과를 종합하여 볼 때 CCh에 의한 Ca^{2+} oscillation에 대한 SNP의 억제 효과는 무스카린성 수용체 혹은 CCh에 의한 내향성 양이온 통로를 억제함으로써 발생하는 것이 아니라 최소한 부분적으로 $InsP_3$ 수용체를 억제함으로써 일어나는 것으로 생각된다.

핵심되는 말: 장관 평활근, carbachol 유발성 양이온 전류, sodium nitroprusside, oscillation