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Regulation of the Contraction Induced by Emptying of Intracellular Ca^{2+} Stores in Cat Gastric Smooth Muscle

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To investigate the mechanism of smooth muscle contraction induced by emptying of intracellular Ca^{2+} stores, we measured isometric contraction and $^{45}Ca^{2+}$ influx. $CaCl_2$ increased Ca^{2+} store emptying- induced contraction in dose-dependent manner, but phospholipase C activity was not affected by the Ca^{2+} store emptying-induced contraction. The contraction was inhibited by voltage-dependent Ca^{2+} channel antagonists dose dependently, but not by TMB-8 (intracellular Ca^{2+} release blocker). Both PKC inhibitors (H-7 and staurosporine) and tyrosine kinase inhibitors (genistein and methyl 2,5-dihydroxycinnamic acid) significantly inhibited the contraction, but calmodulin antagonists (W-7 and trifluoperazine) had no inhibitory effect on the contraction. The combined inhibitory effects of protein kinase inhibitors, H-7 and genistein, together with verapamil were greater than that of each one alone. In Ca^{2+} store-emptied condition, $^{45}Ca^{2+}$ influx was significantly inhibited by verapamil, H-7 or genistein but not by trifluoperazine. However combined inhibitory effects of protein kinase inhibitors, H-7 and genistein, together with verapamil were not observed. Therefore, this kinase pathway may modulate the sensitivity of contractile protein. These results suggest that contraction induced by emptying of intracellular Ca^{2+} stores was mediated by influx of extracellular Ca^{2+} through voltage-dependent Ca^{2+} channel, also protein kinase C and/or tyrosine kinase pathway modulates the Ca^{2+} sensitivity of contractile protein.

Key Words: Emptying of intracellular Ca^{2+} store, Smooth muscle contraction, Calcium channel blocker, Protein kinase C, Tyrosine kinase, $^{45}Ca^{2+}$ influx

INTRODUCTION

A rise in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) is essential for evoking contractile responses in smooth muscle. The rise in $[Ca^{2+}]_i$ is either caused by inositol 1,4,5-trisphosphate (IP_3) released from intracellular Ca^{2+} stores and Ca^{2+} itself (Somlyo et al, 1985; Iino, 1989; Berridge, 1993), or by influx of Ca^{2+} through voltage-dependent Ca^{2+} channels and receptor-operated Ca^{2+} channels. The emptying of the intracellular Ca^{2+} stores open a plasma membrane Ca^{2+} influx (Ohta et al, 1995). This Ca^{2+} influx pathway has been termed capacitative (Putney, 1986 & 1990), which depends on the degree of emptiness of the intracellular Ca^{2+} stores. The mechanism by which the emptying of Ca^{2+} stores can control the permeability of the plasma membrane is still controversial (Tepel et al, 1994). Current hypotheses suggest that emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx (Randriamampita & Tsien, 1993) and this permeation pathway involves direct protein-mediated interaction (Bode & Netter, 1996). Also, role for tyrosine kinase and protein kinase C in the capacitative Ca^{2+} entry was investigated (Petersen & Berridge, 1994; Yule et al, 1994; Petersen & Berridge, 1995). In human platelets, effects of cyclic nucleotides in store-mediated Ca^{2+} entry were examined (Nakamura et al, 1995).

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The increase in intracellular Ca^{2+} concentration results in the activation of a calmodulin-dependent pathway and protein kinase C (Kamm & Stull, 1985), and agonist-induced contraction of smooth muscle is significantly inhibited by calmodulin antagonists and protein kinase C inhibitors (Biancani et al, 1994). Also, many studies have reported that tyrosine kinase is involved in vascular smooth muscle contraction (Tsuda et al, 1991). Therefore, emptying of the intracellular Ca^{2+} store provoke the muscle contraction by extracellular Ca^{2+} influx and this process is correlated with many protein kinase pathway.

In the present study, we have observed that $CaCl_2$ dose-dependently increased the Ca^{2+} store emptying-induced contraction without the stimulation of phospholipase C activity. Ca^{2+} store emptying-induced contraction is inhibited by a variety of Ca^{2+} channel antagonists, protein kinase C inhibitors and tyrosine kinase inhibitors. Therefore, the aim of this study was to investigate the mechanism of contraction induced by emptying of intracellular Ca^{2+} stores in the isolated gastric muscle strips of cats.

METHODS

Materials

Genistein was obtained from Research Biochemicals International (Natick, MA, USA). [^{3}H]-inositol was from Amersham (Bucks, UK) and $^{45}CaCl_2$ was from Du Pont-New England Nuclear (Boston, MA, USA). Acetylcholine bromide, ryanodine, thapsigargin, ethyleneglycol bis (β-aminoethylether) N,N,N', N'-tetraacetic acid (EGTA), verapamil, diltiazem, nifedipine, 3,4,5-trimethoxybenzoic acid 8-(diethylamino) octyl ester (TMB-8), 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), staurosporine, methyl 2,5-dihydroxycinnamic acid (DHC), N-(6-aminohexyl)-5 -chloro-1-naphthalene sulfonamide (W-7), trifluoperazine (TFP) 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). 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, pH 7.4). The Krebs bicarbonate solution was aerated with 95% O_2 - 5% CO_2. Circular muscle strips (1.0, 0.2 cm) were prepared from the fundus, cutting at a right angle to the greater curvature (Mayer et al, 1982).

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 36.5°C and was bubbled with a mixture of 95% O_2 - 5% CO_2 at pH 7.4. Ca^{2+}-free buffer was made by omitting $CaCl_2$ and adding 1 mM EGTA. 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 (FT 03, 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 Ca^{2+}-free solution containing acetylcholine, thapsigargin or ryanodine for 30 min. After Ca^{2+} store emptying, contraction was evoked by adding 2 mM $CaCl_2$. The final concentrations of agonists, antagonists or inhibitors used were achieved by adding 0.01 ml to the chamber. Antagonists and/or inhibitors were administered 5 min before the treatment with $CaCl_2$. Contractile responses to $CaCl_2$ under Ca^{2+} store emptying condition were recorded for 30 min. The maximal amplitude was taken as the contraction measurement for each case.

Preparation of dispersed gastric smooth muscle cells

Muscle cells were isolated from muscle strips of cat stomach as described previously (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 adequate volume of Krebs bicarbonate solution containing 20 mM Na-HEPES (pH 7.4) to measure the $^{45}Ca^{2+}$ influx and inositol phosphates formation.

Measurement of $^{45}Ca^{2+}$ influx

To examine the Ca^{2+} influx, cell suspension was incubated with Ca^{2+}-free solution for 30 min and antagonists or inhibitors were administered 5 min before the treatment with $CaCl_2$. Krebs buffer containing 5 μCi $^{45}Ca^{2+}$ was added to the reaction mixture. The reaction was stopped by the addition of 20 mM EGTA-HEPES and washed three times with 1 mM EGTA-HEPES (Yano et al, 1983). Cells were lysed in the presence of 0.2 N NaOH and radioactivity was measured by liquid scintillation counting (Sipma et al, 1996).

Measurements of inositol phosphates

The isolated cells were incubated in 20 ml of Krebs bicarbonate solution containing Na-HEPES (pH 7.4), 0.1% bovine serum albumin and [^{3}H]inositol (10 μCi/ml) for 3 h at 37°C. After twice washing, the cell reconstituted in 2 ml of Krebs bicarbonate solution containing 20 mM Na-HEPES (pH 7.4), 0.1% bovine serum albumin and 10 mM LiCl. Aliquots of the resuspended cells were incubated for 30 min in Ca^{2+}-free solution and were added by $CaCl_2$. The reaction was terminated by adding 1 ml stop solution (chloroform : methanol : c-HCl = 2 : 1 : 0.1) and then 250 μl of chloroform and 250 μl of distilled water. After centrifugation at 2,000 g for 5 min, 600 μl of supernatant was loaded to Dowex AG1X8 column and the column was washed with 10 ml of distilled water and 20 ml of 60 mM ammonium formate/5 mM sodium borate. [^{3}H]-inositol phosphates were eluted with 4 ml of 1 M ammonium formate/0.1 N formic acid. Radioactivity was determined by liquid scintillation counter and expressed as dpm per mg of protein (Gu et al, 1991). Protein determination was made to correct for differences between preparations using the BCA methods (Smith et al, 1985).

Statistical analysis

The results are represented as means$\pm$SD and analyzed statistically by non-paired Student's t test. The level of significance was set at 5%.

RESULTS

Contraction induced by intracellular Ca^{2+} store emptying

Tonic contraction was induced by 3 mM $CaCl_2$ in Ca^{2+}-free solution containing 1 μM acetylcholine, 100 nM thapsigargin or 10 μM ryanodine. In the subsequent study we added 2.0 g of initial load to the muscle strips and antagonists or inhibitors (X) were treated for 5 min before treatment with $CaCl_2$. Contractile response was observed and then a steady state contraction was maintained and amplitude of maximum contraction was recorded at 30 min after $CaCl_2$ treatment. After 5 min pretreatment with X, change in the amplitude of contraction induced by adding 3 mM $CaCl_2$ was examined and results are expressed as % contraction (b/a$\times$100) at 30 min (Fig. 1A). Fig. 1B showed the dose response curves of $CaCl_2$ and 100% contraction denotes maximum amplitude in steady state after 30 min by addition of 3 mM $CaCl_2$.

Effect of voltage-dependent Ca^{2+} channel blockers on Ca^{2+} store emptying-induced contraction

We examined that the inhibitory effect of voltage-dependent Ca^{2+} channel blockers on the contraction induced by emptying of Ca^{2+} stores (Fig. 2A). In order to evaluate whether the maintenance of steady state contraction required the influx of extracellular Ca^{2+}, three structurally different Ca^{2+} channel blockers, verapamil, nifedipine and diltiazem were used. Verapamil (VP, 10 μM), nifedipine (NP, 10 μM), and diltiazem (DZ, 10 μM) significantly inhibited the emptying-induced contraction by about 50%, but TMB-8 (10 μM, an inhibitor of intracellular Ca^{2+} release) did not affect the contraction.

Effects of protein kinase inhibitors on Ca^{2+} store emptying-induced contraction

To investigate what kind of protein kinase is involved in the contraction induced by Ca^{2+} store emptying of smooth muscle, we used a variety of protein kinase inhibitors. Protein kinase C inhibitors (10 μM H-7, 1 μM staurosporine) and the tyrosine kinase inhibitors (50 μM genistein, 10 μM methyl 2,5-dihydroxycinnamic acid) significantly inhibited the contraction, whereas calmodulin antagonists (50 μM W-7, 30 μM trifluoperazine) did not (Fig. 2B).

This result shows that both extracellular Ca^{2+} influx through voltage-dependent Ca^{2+} channel and protein kinase pathway are required for the contraction induced by Ca^{2+} store emptying in gastric smooth muscle.

$^{45}Ca^{2+}$ influx

In order to evaluate whether the increase of Ca^{2+} concentration is due to the extracellular Ca^{2+} influx, we determined the uptake of extracellular $^{45}Ca^{2+}$ in Ca^{2+} store emptied cells. $^{45}Ca^{2+}$ accumulation was observed after pretreatment of cells with verapamil, H-7, genistein or trifluoperazine. After emptying of

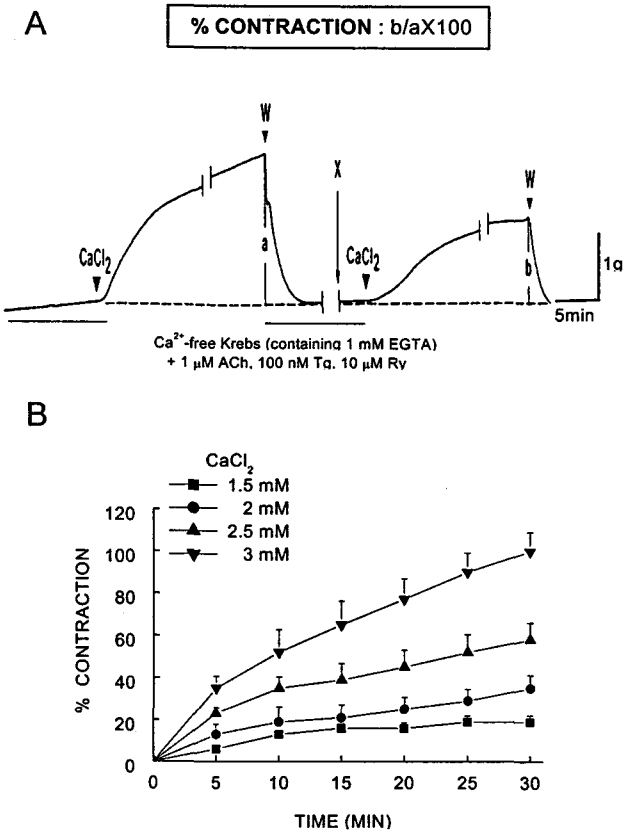

Fig. 1. (A) Isometric contractions of cat gastric smooth muscle by $CaCl_2$ in Ca^{2+}-free solution. Emptying of Ca^{2+} store was performed for 30 min in Ca^{2+}-free Krebs (1 mM EGTA) containing 1 μM ACh, 100 nM thapsigargin (Tg) or 10 μM ryanodine (Ry). After 5 min pretreatment with antagonists (X), change in the amplitude of contraction induced by adding 3 mM $CaCl_2$ was examined. Results are expressed as % contraction (b/a $\times$ 100) at 30 min. (B) Dose responses of Ca^{2+} store emptying-induced contraction to extracellular Ca^{2+} concentration. Muscle strip was incubated for 30 min in Ca^{2+}-free Krebs containing 1 mM EGTA and 1 μM ACh, and then measured isometric contraction. Adding $CaCl_2$ in a dose dependent manner evoked emptying- induced contraction. Numerals on the top of the figure indicate the concentration of $CaCl_2$ (mM). Results are means $\pm$ S.D. of five experiments.

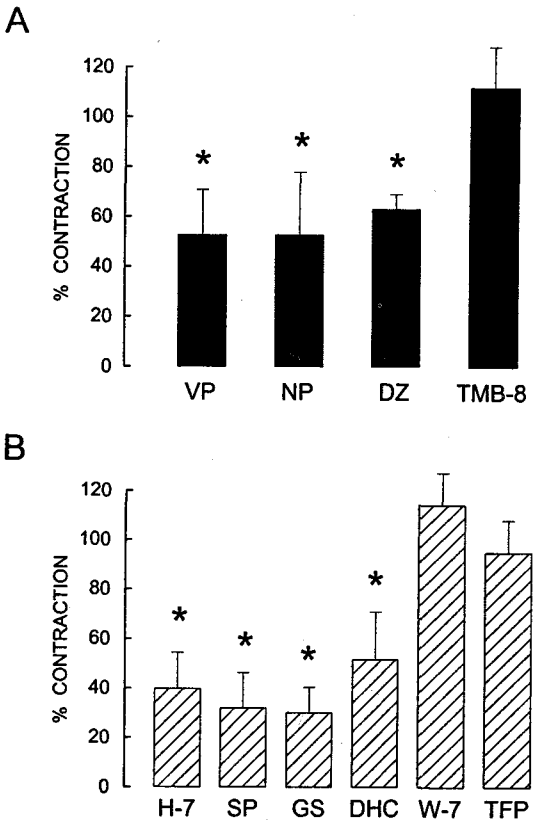

Fig. 2. (A) The inhibitory effects of Ca^{2+} channel blockers on Ca^{2+} store emptying-induced contraction produced by addition of 3 mM $CaCl_2$. Verapamil (VP, 10 μM), nifedipine (NP, 10 μM), and diltiazem (DZ, 10 μM) significantly inhibited the emptying-induced contraction by about 50%. TMB-8 (10 μM, an inhibitor of intracellular Ca^{2+} release) did not affect the contraction. All chemicals were incubated for 5 min prior to $CaCl_2$ addition. Results are means $\pm$ S.D. from seven experiments. (B) The inhibitory effects of protein kinase inhibitors on the contraction induced by 3 mM $CaCl_2$ in the Ca^{2+} store-emptied condition. Both PKC inhibitors (10 μM H-7 and 1 μM staurosporine (SP)) and tyrosine kinase inhibitors (10 μM genistein (GS) and 10 μM DHC) significantly inhibited the contraction, but calmodulin antagonists (50 μM W-7 and 30 μM TFP) did not. All chemicals were incubated for 5 min prior to $CaCl_2$ addition. Results are means $\pm$ S.D. from ten experiments. *$P < 0.05$ vs. Ca^{2+} store emptying-induced contraction without pretreatment with blockers or inhibitors (non-pretreated control).

Ca^{2+} stores, influx of $^{45}Ca^{2+}$ was stimulated time dependently (Fig. 3A). 10 μM verapamil, 10 μM H-7 and 50 μM genistein significantly inhibited $^{45}Ca^{2+}$ influx compared with that before treatment, but 30 μM TFP did not (Fig. 3B).

Combined effects of voltage-dependent Ca^{2+} channel blockers and kinase inhibitors on contraction and $^{45}Ca^{2+}$ influx

The combined inhibitory effects of protein kinase inhibitors, H-7 and genistein, together with verapamil on contraction induced by Ca^{2+} stores emptying were greater than that of either one alone (Fig. 4A). However combined inhibitory effects of protein kinase inhibitors, H-7 and genistein, together with verapamil on $^{45}Ca^{2+}$ influx were not observed (Fig. 4B). From the above results, this kinase pathway may modulate the sensitivity of contractile protein.

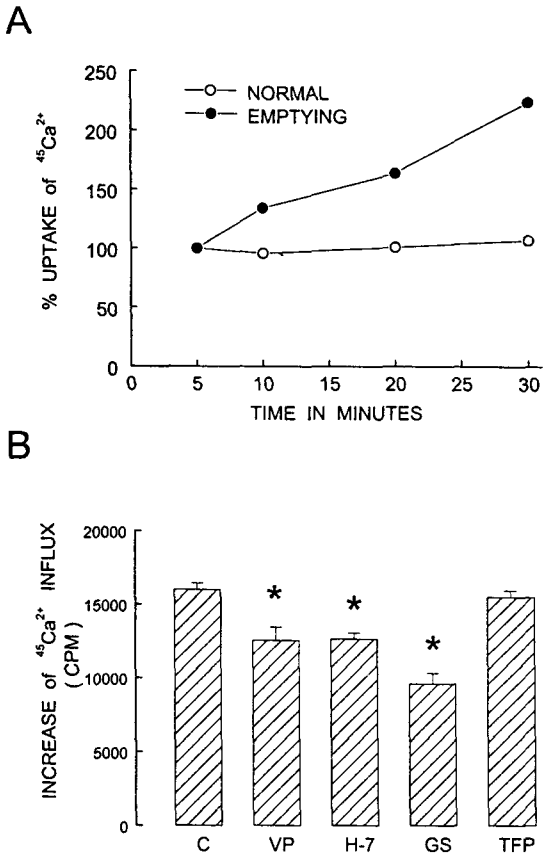

Fig. 3. Measurement of $^{45}Ca^{2+}$ influx, (A) Smooth muscle cells were incubated for 30 min in normal Krebs buffer (○) or Ca^{2+}-free Krebs containing 1 mM EGTA and 1 μM ACh (●). After incubation, the cells were incubated with 5 μCi $^{45}CaCl_2$ at 5 min interval for 30 min at 37°C. Reaction was stopped with 0.2 N NaOH. (B) Effects of Ca^{2+} channel blocker and kinase inhibitors on $^{45}Ca^{2+}$ influx were examined. Verapamil (VP, 10 μM), H-7 (10 μM) and genistein (GS, 50 μM) significantly inhibited $^{45}CaCl_2$ influx compared with that before treatment (C), but TFP (30 μM) did not. All chemicals were incubated for 5 min prior to 5 μCi $^{45}Ca^{2+}$ addition. *$P<0.05$ vs. non-pretreated control (C).

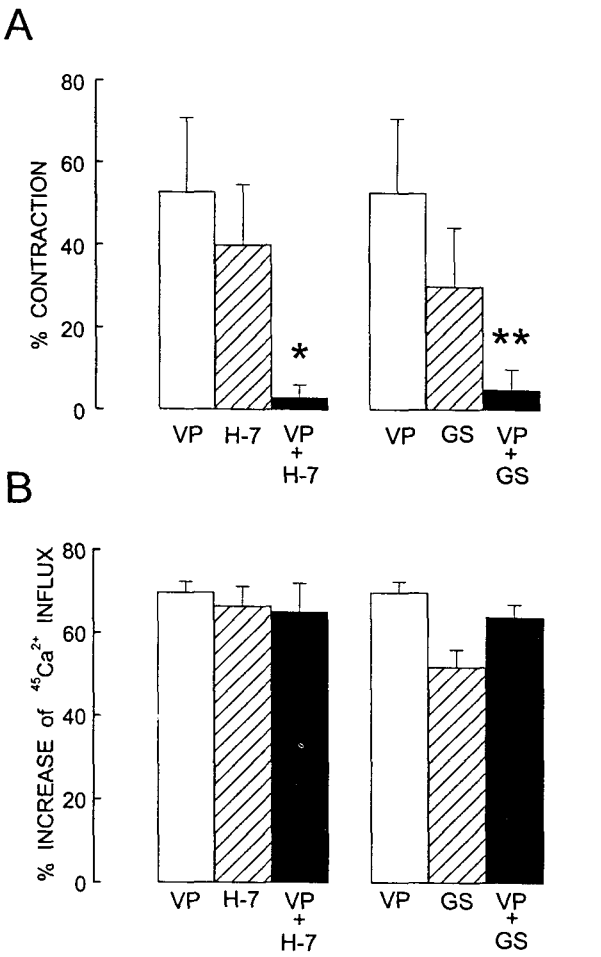

Fig. 4. (A) The combined effects of protein kinase inhibitors and verapamil on the Ca^{2+} store emptying-induced contraction. The combined inhibitory effects of protein kinase inhibitors, H-7 (10 μM) and genistein (GS, 10 μM) together with verapamil (VP, 10 μM) were significantly greater than the effect of each one, respectively. All chemicals were incubated for 5 min prior to $CaCl_2$ addition. Results are means ± S.D. from eight experiments. (B) The combined effects of protein kinase inhibitors and verapamil on the $^{45}CaCl_2$ influx, *$P<0.05$ vs. VP or H-7 alone, **$P<0.05$ vs. VP or GS alone.

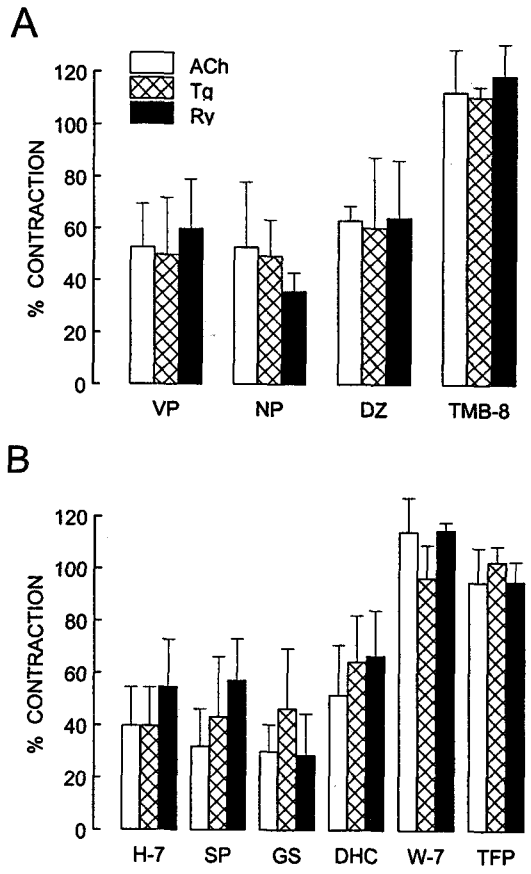

Fig. 5. The effects of Ca^{2+} antagonists (A, verapamil (VP), nifedipine (NP), diltiazem (DZ), TMB-8) and protein kinase inhibitors (B, H-7, staurosporine (SP), genistein (GS), DHC, W-7, TFP) in the Ca^{2+} store emptying-induced contraction. There are not any significant differences among the addition of acetylcholine (ACh), thapsigargin (Tg) and ryanodine (Ry) in the Ca^{2+} store emptying in terms of the emptying-induced contraction following $CaCl_2$ addition.

Phospholipase C activity in gastric smooth muscle cells by Ca^{2+} store emptying-induced contraction

Acetylcholine significantly increased the inositol triphosphates levels in normal gastric muscle cells, but the formation of inositol triphosphates was not affected by the Ca^{2+} store emptying-induced contraction (Table 1). These results indicate that contraction induced by emptying of Ca^{2+} store was evoked without the stimulation of phospholipase C activity.

Table 1. Formation of [^{3}H]inositol phosphates following $CaCl_2$ addition in Ca^{2+} store-emptied state

Treatments	Doses	[^{3}H]inositol phosphates (% increase)
Control		0
ACh	0.1 μM	33.3%*
	1 μM	48.2%*
	10 μM	75.3%*
Emptying control		0
$CaCl_2$	4 mM	−5%
	6 mM	−0.2%
	8 mM	1.4%
	10 mM	1.2%

Control: without emptying of Ca^{2+} store, Emptying control: Intracellular Ca^{2+} store emptying was induced by addition of 100 nM thapsigargin in Ca^{2+}-free Krebs solution containing 1 mM EGTA. *: $P<0.05$ vs. control

DISCUSSION

Many cellular processes are mediated by an elevation of the free Ca^{2+} concentration. Cytosolic Ca^{2+} can be elevated by increased Ca^{2+} influx across the plasma membrane, by Ca^{2+} release from intracellular Ca^{2+} stores, or by a combination of both. In cat gastric smooth muscle strips, $CaCl_2$ dose-dependently increased the contraction induced by Ca^{2+} store emptying without the stimulation of phospholipase C activity. These results suggest that emptying of intracellular Ca^{2+} stores regulates the contraction, but mechanism of the contraction remains unknown.

In vascular smooth muscle, L-type Ca^{2+} channel activator, Bay K 8644, activates the agonist-induced contraction in Ca^{2+}-free condition (Low et al, 1992). However, Ohta et al (1995) reported that emptying of intracellular Ca^{2+} stores may activate Ca^{2+} influx not associated with voltage-dependent Ca^{2+} channel in the rat ileal muscle. Consequently, the mechanism of the contraction induced by Ca^{2+} store emptying may be tissue-specific.

To determine the involvement of the voltage-dependent Ca^{2+} channel (VDCC) in the influx of extracellular Ca^{2+}, three structurally different VDCC antagonists, verapamil, nifedipine or diltiazem were used. All of the VDCC antagonists dose dependently inhibited the contraction induced by Ca^{2+} store emptying,

but the inhibitory effects of VDCC antagonists at 10 μM did not exceed 50%. Therefore some kinds of Ca^{2+} channel, other than VDCC, may be involved in the influx of extracellular Ca^{2+}. However, TMB-8, a blocker of intracellular Ca^{2+} release, did not affect the contraction.

A variety of protein kinase including those of the calmodulin-dependent pathway, protein kinase C and tyrosine kinase play an important role in the regulation of smooth muscle contraction. Protein kinase C inhibitors (H-7 and staurosporine) and tyrosine kinase inhibitors (genistein and DHC) significantly inhibited the contraction induced by Ca^{2+} store emptying, but calmodulin antagonists (W-7 and trifluoperazine) did not inhibit the contraction at all. These results suggest that both protein kinase C and tyrosine kinase pathway appear to be involved in the Ca^{2+} store emptying-induced contraction.

As mentioned above, the inhibitory effects of VDCC antagonists at maximal concentration did not exceed 50%. Therefore we examined whether there is interaction between Ca^{2+} influx through VDCC and other regulatory process. Many investigators have reported that there is a close interaction between VDCC and protein kinase pathway (Litten et al, 1987; Fish et al, 1988; Wijetunge et al, 1992). Our results showed that combined treatment with verapamil and protein kinase inhibitors completely inhibited the contraction induced by Ca^{2+} store emptying. Whether the contraction and the increase of Ca^{2+} concentration are due to the extracellular Ca^{2+} influx, we determined the uptake of extracellular $^{45}Ca^{2+}$ in Ca^{2+} store emptied cells. $^{45}Ca^{2+}$ accumulation was reduced after pretreatment of cells with verapamil, H-7 or genistein. It is of interest that combined inhibitory effects of protein kinase inhibitors, H-7 and genistein, together with verapamil on $^{45}Ca^{2+}$ influx were not observed. From the above results, this kinase pathway may modulate the sensitivity of contractile protein rather than VDCC.

Contraction induced by emptying of intracellular Ca^{2+} stores was evoked without the stimulation of phospholipase C activity, whereas contraction induced by acetylcholine required the stimulation of phospholipase-C. Since Sim et al (1997) reported that the involvement of protein kinase in regulation of the contraction may be agonist-dependent, we measured the contraction induced by emptying of intracellular Ca^{2+} stores with various agonists. But there were not significant differences from the Ca^{2+} store-emptied condition by acetylcholine, thapsigargin or ryanodine in the Ca^{2+} store emptying-induced contraction.

Therefore, it is concluded that contraction induced by emptying of intracellular Ca^{2+} stores was mediated by influx of extracellular Ca^{2+} through VDCC without the stimulation of phospholipase C activity, also protein kinase C and/or tyrosine kinase pathway modulates the Ca^{2+} sensitivity of contractile protein.

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