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The Involvement of Protein Kinase C and Tyrosine Kinase in Vanadate-induced Contraction

Sang Soo Sim and Chang Jong Kim

Department of Pathophysiology, College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea

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Gastric smooth muscle of cats was used to investigate the involvement of protein kinase in vanadate-induced contraction. Vanadate caused a contraction of cat gastric smooth muscle in a dose-dependent manner. Vanadate-induced contraction was totally inhibited by 2 mM EGTA and 1.5 mM $LaCl_3$ and significantly inhibited by 10 μM verapamil and 1 μM nifedipine, suggesting that vanadate-induced contraction is dependent on the extracellular Ca^{2+} concentration, and the influx of extracellular Ca^{2+} was mediated through voltage-dependent Ca^{2+} channel. Both protein kinase C inhibitor and tyrosine kinase inhibitor significantly inhibited the vanadate-induced contraction and the combined inhibitory effect of two protein kinase inhibitors was greater than that of each one. But calmodulin antagonists did not have any influence on the vanadate-induced contraction. On the other hand, both forskolin (1 μM) and sodium nitroprusside (1 μM) significantly inhibited vanadate-induced contraction. Therefore, these results suggest that both protein kinase C and tyrosine kinase are involved in the vanadate-induced contraction which required the influx of extracellular Ca^{2+} in cat gastric smooth muscle, and that the contractile mechanism of vanadate may be different from that of agonist binding to its specific receptor.

Key words : Calcium channel antagonist, Protein Kinase C, Smooth muscle contraction, Tyrosine kinase

INTRODUCTION

Smooth muscle contraction in response to neurotransmitters and hormones is closely related to an increase in the concentration of intracellular free Ca^{2+} via a release of Ca^{2+} from intracellular stores and an influx of extracellular Ca^{2+} as well as the phosphorylation of contractile protein. The influx of extracellular Ca^{2+} by agonists is mediated via voltage-dependent Ca^{2+} channel and receptor-operated Ca^{2+} channel (Ozaki *et al.*, 1991; Ohta *et al.*, 1995), and the release of intracellular Ca^{2+} is mediated via inositol trisphosphate-induced Ca^{2+} release and Ca^{2+}-induced Ca^{2+} release from intracellular calcium stores (Duncan *et al.*, 1987; Sim *et al.*, 1993). This could be supported by various observations that Ca^{2+} channel antagonists significantly inhibited the contractions induced by agonists in vascular and visceral smooth muscle (Chijiiwa *et al.*, 1991; Hagiwara *et al.*, 1993). A variety of protein kinases, such as protein kinase C, calmodulin-dependent pathway and tyrosine kinase, are also associated with smooth muscle contraction. Visceral and vascular smooth muscle contractions induced by agonists were inhibited by protein kinase C inhibitor (Yang and Black, 1995), calmodulin antagonists (Hillemeier *et al.*, 1991) and tyrosine kinase inhibitor (Jinsi and Deth, 1995).

Vanadate as an essential trace element is present in all living tissues of animals and humans (Josephson and Cantley, 1977). A large number of studies have shown that vanadate caused contraction of vascular and visceral smooth muscle. Unlike neurotransmitters and hormones, the mechanism underlying the effect of vanadate on smooth muscle contraction is uncertain but may be related to (1) the inhibition of ATPase which resulted in the increased of intracellular Ca^{2+} concentration (Sunano *et al.*, 1992), (2) the inhibition of tyrosine phosphatase which caused the increase of phosphotyrosine level of contractile protein (Laniyonu *et al.*, 1994), and (3) the stimulation of G-protein which brought about the activation of phospholipase C (Dreskin, 1995). The aim of the present study was to investigate which kind of protein kinase and Ca^{2+} source are involved in vanadate-induced contraction.

Correspondence to: Sang Soo Sim, College of Pharmacy, Chung-Ang University, 221, Huksuk-Dong, Dongjak-ku, Seoul 156-756, Korea

MATERIALS AND METHODS

Materials

Vanadate, verapamil, nifedipine, $LaCl_3$, tetrodotoxin, atropine, 8-(N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8), EGTA, staurosporine, H-7, genistein, methyl 2,5-dihydroxycinnamate, W-7, trifluoperazine, forskolin and sodium nitroprusside were obtained from Sigma (St. Louis, MO, USA). All other reagents used were of analytical grade.

Preparation of gastric smooth muscle strips

Cats of either sex (1.8~2.6 kg) were anesthetized with 20% urethane (5 ml/kg, intraperitoneal) following 16 h of fasting but with water ad libitum. The whole stomach was removed from each cat, and the mucous membrane was peeled off in ice-cold Krebs bicarbonate solution (mM: 120.8 NaCl, 4.5 KCl, 15.5 $NaHCO_3$, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4 and 5.6 dextrose). The Krebs bicarbonate solution was aerated with 95% O_2-5% CO_2 until the pH was 7.4. Circular muscle strips (1.0×0.2 cm) were prepared from fundus, cutting at a right-angle to the greater curvature (Sim *et al.*, 1997).

Measurement of contractile response

The circular muscle strips were used to measure the contraction in a cylinder-shaped muscle chamber (10 ml capacity) filled with Krebs bicarbonate solution. The solution of the chamber was kept at 37°C and was bubbled with a mixture of 95% O_2-5% CO_2 at pH 7.4. To record the isometric contraction, the lower end of the muscle strips was anchored to a steel hook and the upper end to a force transducer (FT03, Grass Instruments, Quincy, MA, USA) connected to a Grass 7E polygraph. The muscle preparation was loaded with a tension of 2.0 g and allowed to equilibrate with the solution for 30 min. The final concentrations of agonist, antagonists or inhibitors used were achieved by adding 0.01 ml of each stock solution to the chamber. The isometric contraction was induced by 1~100 μM vanadate. Vanadate slowly caused the tonic contraction which maintained for more than 2 h. To investigate the effects of Ca^{2+} antagonists and protein kinase inhibitors on vanadate-induced contraction, the relaxation induced by Ca^{2+} antagonists or protein kinase inhibitors was measured for 10 min and finally complete relaxation was induced by adding 2 mM EGTA. The relaxation induced by each agent was expressed as % inhibition that meant percentage of the maximum relaxation obtained after treatment with 2 mM EGTA.

Statistical analysis

The results are presented as means±S.D. and analyzed statistically by analysis of variance (ANOVA), and differences between groups were determined with Neuman-Keuls test. The level of significance was set at 5%.

RESULTS

Effect of vanadate on smooth muscle contraction

As shown in Fig. 1, vanadate slowly caused smooth muscle contraction in a dose-dependent manner. Vanadate-induced contraction reached to peak level at 20 min and maintained for more than 2 h. To investigate that vanadate-induced contraction was related to the release of neurotransmitters from myenteric plexus, we measured vanadate-induced contraction in the presence of various neurotransmitter antagonists. Vanadate-induced contraction was not affected by 1 μM atropine, 1 μM hexamethonium, 1 μM phentolamine, 1 μM propranolol and 1 μM tetrodotoxin, indicating that the contractile mechanism of vanadate may be myogenic effect but not neurogenic effect (Fig. 2).

The effects of Ca^{2+} channel antagonists

As shown in Fig. 3, EGTA inhibited vanadate-induced contraction dose-dependently and completely inhibited at a concentration of 2 mM, showing that vanadate-induced contraction is wholly dependent on the extracellular Ca^{2+} concentration. $LaCl_3$ also inhibited vanadate-induced contraction in a dose-dependent manner and completely inhibited at a concentration of 1.5 mM, suggesting that vanadate-induced contraction is dependent on the influx of extracellular Ca^{2+}. Since vanadate-induced contraction required the influx of extracellular Ca^{2+}, we used two structurally different

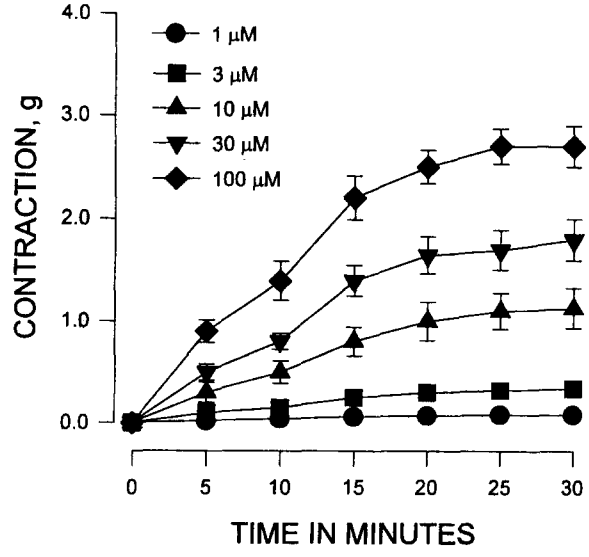

Fig. 1. Dose-response curves of gastric smooth muscle contraction to vanadate. Vanadate dose-dependently caused contraction. Results are means±S.D. of eight experiments.

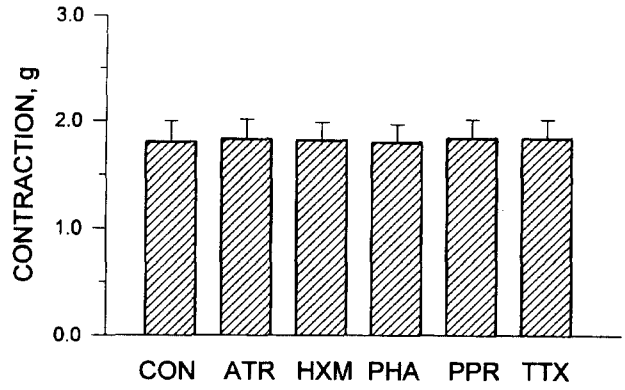

Fig. 2. Effects of neurotransmitter antagonists on vanadate-induced contraction. After incubation with atropine (ATR, 1 μM), hexamethonium (HXM, 1 μM), phentolamine (PHA, 1 μM), propranolol (PPR, 1 μM), and tetrodotoxin (TTX, 1 μM) for 5 min, isometric contraction was induced by 30 μM vanadate. Control (CON) was obtained after addition of distilled water. Antagonists did not have any influence on the vanadate-induced contraction. Results are means±S.D. of eight experiments.

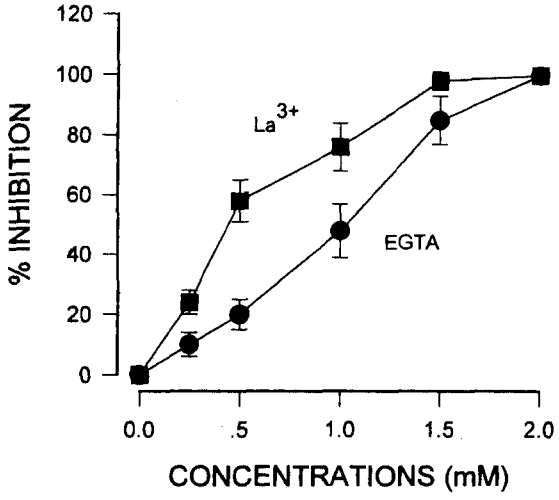

Fig. 3. Effects of EGTA and La^{3+} on vanadate-induced contraction. EGTA and La^{3+} dose-dependently inhibited the contraction. This result suggests that the contraction depends on extracellular calcium concentration. Results are means± S.D. from eight experiments. Responses were recorded 10 min after addition of EGTA or La^{3+} at concentrations of 0.25, 0.5, 1.0, 1.5 and 2.0 mM.

Ca^{2+} channel antagonists, verapamil and nifedipine (Catterall and Striessnig, 1992) to investigate whether this influx was mediated via voltage-dependent Ca^{2+} channel (VDCC). Both verapamil (10 μM) and nifedipine (1 μM) significantly inhibited vanadate-induced contraction by 56% and 58%, respectively (Fig. 4). TMB-8, a blocker of intracellular Ca^{2+} release, did not affect vanadate-induced contraction.

Effects of protein kinase inhibitors

To investigate which kind of protein kinase is involved in vanadate-induced contraction, we used a variety of protein kinase inhibitors, protein kinase C inhibitors (H-7 and staurosporine), calmodulin antagonists (W-7 and trifluoperazine) and tyrosine kinase inhibitors (genistein and methyl 2,5-dihydroxycinnamate). As shown in Fig. 5, H-7 (10 μM) and staurosporine (1 μM) significantly inhibited vanadate-induced contraction by 63% and 54%, respectively. Genistein (20 μM) and methyl 2,5-dihydroxycinnamate (10 μM) also sig-

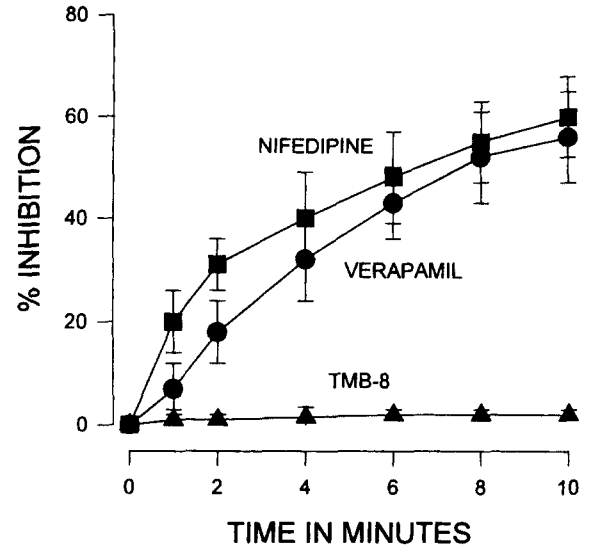

Fig. 4. Effects of calcium antagonists on 30 μM vanadate-induced contraction. Voltage-dependent Ca^{2+} channel blockers, verapamil (10 μM) and nifedipine (1 μM) significantly inhibited the contraction, but TMB-8 (10 μM), a blocker of intracellular Ca^{2+} release, did not inhibit the contraction at all. Results are means±S.D. from eight experiments.

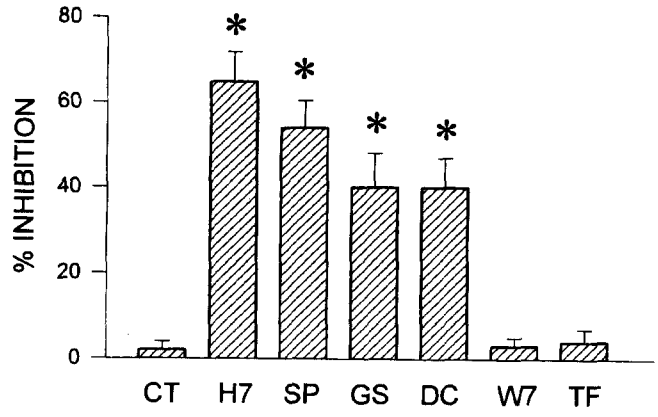

Fig. 5. Effects of protein kinase inhibitors on 30 μM vanadate-induced contraction. Protein kinase C inhibitors, 10 μM H7 and 1 μM staurosporine (SP), and tyrosine kinase inhibitors, 20 μM genistein (GS) and 10 μM methyl 2,5-dihydroxycinnamate (DC), significantly inhibited the contraction, but calmodulin antagonists, 50 μM W7 and 30 μM trifluoperazine (TF), did not. Control (CT) was obtained after addition of 0.1% dimethylsulfoxide. Results are means±S.D. from eight experiments. Responses were recorded 10 min after addition of protein kinase inhibitors. *$P<0.05$ vs. control (CT).

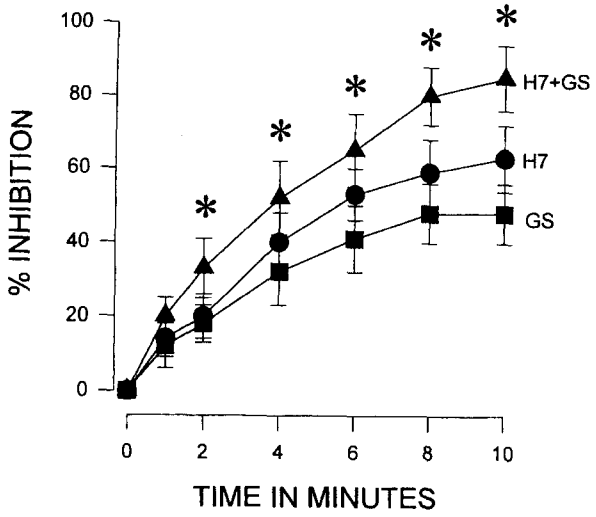

Fig. 6. The combined effects of H7 and genistein (GS) on 30 μM vanadate-induced contraction. The combined inhibitory effect of 10 μM H7 and 20 μM GS was significantly greater than that of H7 or GS alone, suggesting that both protein kinase C and tyrosine kinase are involved in vanadate-induced contraction. Results are means±S.D. from eight experiments. *P<0.05 vs. H-7 or GS.

nificantly inhibited the contraction by 41% and 40%, respectively. But W-7 (50 μM) and trifluoperazine (30 μM) did not inhibit the contraction at all. Combined inhibitory effect of H-7 and genistein was significantly more potent than the effect of H-7 or genistein alone (Fig. 6).

Effects of forskolin and sodium nitroprusside

To investigate the effects of cyclic nucleotides on vanadate-induced contraction, we used forskolin as an adenylyl cyclase activator and sodium nitroprusside as a guanylyl cyclase activator. Both forskolin (1 μM) and sodium nitroprusside (1 μM) significantly inhibited vanadate-induced contraction. The complete inhibition by sodium nitroprusside rapidly occurred within 2 min, whereas the one by forskolin slowly did at more than 10 min (Fig. 7).

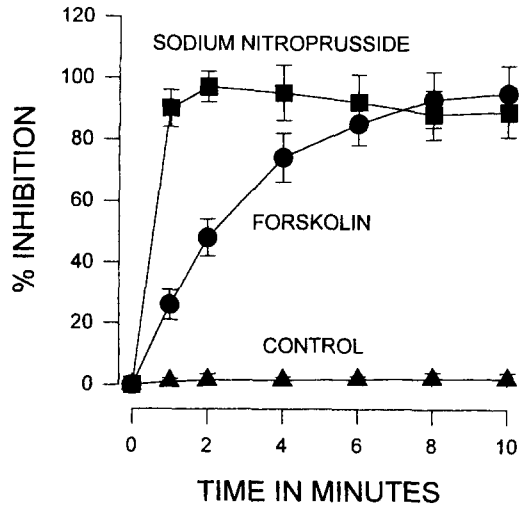

Fig. 7. Effects of forskolin and sodium nitroprusside on 30 μM vanadate-induced contraction. Foskolin (1 μM) and sodium nitroprusside (1 μM) significantly inhibited the contraction. Results are mean±S.D. from eight experiments.

DISCUSSION

Vanadate caused contraction through direct action on gastric smooth muscle and the contractile mechanism of vanadate was related to the influx of extracellular Ca^{2+} and the involvement of protein kinase C and tyrosine kinase. Gastric muscle strips used in this experiment contained myenteric plexus between muscle layers releasing various neurotransmitters on stimulation. Since one of actions of vanadate is the inhibition of ATPase (Sunano *et al.*, 1992), it can not be ruled out the possibility that some neurotransmitters are released from myenteric plexus by vanadate. However, vanadate-induced contraction did not alter at all in the presence of various neurotransmitter antagonists or tetrodotoxin, which suggests that the contractile mechanism of vanadate is due to the direct action on smooth muscle rather than on myenteric plexus.

Vanadate-induced contraction was totally inhibited by 2 mM EGTA or 1.5 mM $LaCl_3$, and significantly inhibited by voltage-dependent Ca^{2+} channel antagonist, but was not inhibited by TMB-8, a blocker of intracellular Ca^{2+} release. This data is in accord with previous report that vanadate caused the influx of extracellular Ca^{2+} via voltage-dependent Ca^{2+} channel in rabbit ileal smooth muscle (Candura *et al.*, 1994). These results suggest that vanadate-induced contraction is dependent on extracellular Ca^{2+} influx and is independent on intracellular Ca^{2+} release. These inhibitory effects of Ca^{2+} channel antagonists on vanadate-induced contraction were similar to those on KCl-induced contraction and were different from those on acetylcholine-induced contraction (Sim *et al.*, 1997).

To investigate which kind of protein kinase is involved in vanadate-induced contraction, we used protein kinase C inhibitors (10 μM H-7 and 1 μM staurosporine), tyrosine kinase inhibitors (20 μM genistein and 10 μM methyl 2,5-dihydroxycinnamate) and calmodulin antagonists (50 μM W-7 and 30 μM trifluoperazine). The concentrations of protein kinase inhibitors were widely used to investigate the effect of their specific action (Hillemeier *et al.*, 1991; Jinsi and Deth, 1995; Yang and Black, 1995). Both protein kinase C inhibitors and tyrosine kinase inhibitors significantly inhibited vanadate-induced contraction but calmodulin antagonists did not. Salvo *et al.* (1993) have reported that vanadate caused smooth muscle contraction via increase of protein phosphotyrosine

level, which was inhibited by genistein. However, little has been reported regarding the involvement of protein kinase C in vanadate-induced contraction. The combined effect of protein kinase C inhibitor and tyrosine kinase inhibitor were significantly more potent than that of each one. This data suggests that both protein kinase C and tyrosine kinase are involved in vanadate-induced contraction. The involvement of protein kinase in vanadate-induced contraction was also similar to that in KCl-induced contraction but different from that in acetylcholine-induced contraction (Sim *et al.*, 1997). Taken together, it is suggested that the contractile mechanism of vanadate may be different from that of agonists binding to their specific receptor.

On the other hand, the increase of cellular cyclic nucleotide level caused relaxation of visceral smooth muscle. Forskolin as an adenylyl cyclase activator (Seamon and Daly, 1981) and sodium nitroprusside as a guanylyl cyclase activator (Ignarro *et al.*, 1986) increased the level of cAMP and cGMP, respectively. Both forskolin and sodium nitroprusside significantly inhibited vanadate-induced contraction, showing that the contractile mechanism of vanadate was blocked by cAMP- and cGMP-dependent pathway.

In summary, it is suggested that both protein kinase C and tyrosine kinase are involved in vanadate-induced contraction which required the influx of extracellular Ca^{2+} in cat gastric smooth muscle, and that the contractile mechanism of vanadate may be different from that of agonist binding its specific receptor.

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