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# The Excitatory Mechanism of Substance P in the Antral Circular Muscle of Guinea Pig Stomach

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=ABSTRACT=

**This study was carried out to elucidate the excitatory mechanisms of Substance P in the antral circular muscle, using isometric contraction recording, conventional microelectrode method and whole-cell patch clamp technique. Substance P produced tonic and phasic contractions in a dose-dependent manner and depolarized membrane potential with increased amplitude of slow waves in muscle strips. Voltage-dependent $Ca^{2+}$ currents were increased by the application of Substance P from a holding potential of −60 mV to 50 mV in 10 mV steps and this effect was blocked by the addition of an antagonist.**

**Also Substance P increased transient and spontaneous oscillatory $K^+$ outward currents. The enhanced outward currents were abolished by apamin in dispersed single cells. These results suggest that the depolarization of membrane potential by Substance P activates voltage-dependent $Ca^{2+}$ channels, which represents an excitatory response in the antral circular muscle and led to an increase in $Ca^{2+}$ activated $K^+$ channels.**

**Key Words:** Slow wave, Antral myocytes, Substance P, Voltage-dependent $Ca^{2+}$ currents, $Ca^{2+}$ activated $K^+$ currents

## INTRODUCTION

The spontaneous contractions of gastrointestinal smooth muscle are initiated by the electrical slow waves which are modulated by the intrinsic and extrinsic autonomic nervous systems as well as by the circulating hormones and drugs (Demol et al, 1989). Autonomic nervous systems composed of not only sympathetic and parasympathetic nerves but also non-adrenergic non-cholinergic (NANC) nerves (Burnstock G, 1986; Komori & Suzuki, 1986; Ito et al, 1988). Substance P is a well known non-adrenergic, non-cholinergic excitatory neurotransmitter in mammalian visceral smooth muscle. The ability of Substance P to strongly stimulate gastrointestinal smooth muscle suggests a significant role in the regulation of gastrointestinal motor function. It is reported that Substance P containing neurons are widely distributed in the muscle layer, especially in the circular muscle of rat and guinea-pig stomach and Substance P is secreted at the pylorus (Uvana-Wallensten, 1978). Also, Sub-stance P is secreted from enterochromaffine cells (Bartho & Holzer, 1985). Nevertheless, its function and mecha-

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nisms in the gastrointestinal system are not fully comprehended.

The present experiments were undertaken to clarify the excitatory mechanisms of Substance P in the antral circular muscle of guinea-pig stomach.

## MATERIALS AND METHODS

Guinea pigs of either sex, weighing about 250-300 g, were stunned and bled. The whole stomach was isolated and cut in the longitudinal direction along the lesser curvature. The contents of the stomach were removed and mucosal layers were separated from the muscle layers in an oxygenated phosphate-buffered Tyrode solution (NaCl 147, KCl 4, $MgCl_2.6H_2O$ 1.05, $CaCl_2.2H_2O$ 2, $NaH_2PO_42H_2O$ 0.42, $Na_2HPO_4.12H_2O$ 1.81, glucose 5. 5 mM, pH 7.35 at room temperature). Strips of circular muscle (2 mm wide, 10 mm long) were isolated together with the longitudinal layer.

Mechanical contractions were recorded in a vertical chamber which had a capacity of 100 ml. Before the main experiment, the strip was allowed to recover in an oxygenated tris-buffered Tyrode solution NaCl 147, KCl 4, $MgCl_26H_2O$ 1.05, $CaCl_22H_2O$ 2, tris. HCl 5, glucose 5.5 mM, pH 7.35) for 1 hour at 35°C. Isometric contractions were recorded through a force transducer (Harvard, USA) connected to a physiograph (Device. The optimal length of the strip was determined by length-tension curve of either spontaneous contractions or contractions evoked by electrical field stimulation.

The electrical and mechanical responses were recorded simultaneously using a conventional glass capillary microelectrode method in a horizontal chamber which had a capacity of 2 ml. In the chamber the strip was pinned out at one end with tiny pins onto a rubber plate and was connected to a Grass FT-03 force transducer at the other free end. The strip was perfused constantly at a rate of 2-3 ml/min, with tris buffered Tyrode solution that was bubbled with 100% $O_2$ and kept at 35 0C. Electrical responses of smooth muscle cells were recorded using a glass microelectrode of tip resistance 40-50 M and filled with 3 M KCl.

To isolate single smooth muscle cells, circular muscle strips were separated from the longitudinal muscle layer and small segments were made in the $Ca^{2+}$-free PSS (physiological salt solution) for 30 mins at room temperature. Small segments were incubated for 20-30 min in $Ca^{2+}$-free PSS containing 0.1% collagenase, 0.1% trypsin inhibitor, and 0.2% bovine serum albumin at 35°C. After complete digestion, single cells were dispersed by gentle agitation with a glass pipette in the Krafts-Brühe (KB) solution. Isolated gastric myocytes were kept in KB medium at 4°C. All experiments were carried out within 1 hour of cell harvest.

The isolated cells were transfered to the bath on the inverted microscope (Olympus IMT-2). The chamber was perfused with PSS at a velocity of 2-3 ml/min. Standard patch clamp recording technique were used (Hamill et al, 1980). Glass electrodes of resistance of 2-5 M were used. An Axopatch-clamp amplifier (Axon instrument) was used to record membrane currents. The data was displayed on a digital oscilloscope (Phillips, PM3350), pen recorder (Gould, Recoder 220) and stored on a videotape recorder (Victor, BR-6400) with a pulse code modulator (NF, RP-880). All experiments were performed at room temperature

**Solution:** $Ca^{2+}$-free PSS contained (mM) NaCl 134.8, KCl 6.2, $CaCl_2$ 0,glucose 12.2, HEPES 0.4 and pH was adjusted to 7.3 by tris. PSS contained 2.3 mM $CaCl_2$ in the $Ca^{2+}$-free PSS. KB solution contained (in mM) L-glutamate 50, KCl 50, Taurine 20, $KH_2PO_4$ 20, $MgCl_2$ 3, glucose 10, HEPES 10, EGTA 0.5 and pH was adjusted to 7.3 by KOH. Electrode solution consisted of (in mM) $K^+$-aspartate 110, Mg-ATP 5, di-Tris-creatine phosphate 5, KCl 20, $MgCl_2$ 1, ethylen glycol-bis (-aminoethyl ether)-N,N,N',N'-tetraacetic

acid(EGTA) 0.1, HEPES 5, pH 7.4. For studies in which $K^+$ currents were blocked, internal solution was used with (in mM) Cs-aspartate 110, Mg-ATP 5, di-tris-creatine phosphate 2.5, $MgCl_2$ 1, HEPES 0.1, tetra-ethylammonium (TEA)-Cl 20, EGTA 5, pH 7.4

### Drugs

The following drugs were used. Apamin (Sigma chemical), Atropine (Sigma chemical) Guanethidine sulfate (Tokyo Kaesi) Substance P (Sigma chemical) Substance P antagonist (D-Pro[2], D-Phe[7], D-Trp[9]) (Sigma chemical), Tetrodotoxin (Sigma chemical).

## RESULTS

### Effects of Substance P on the contractility of the antral circular muscle

The dose-response relationship of Substance P and spontaneous contraction of the antral circular muscle strip are presented in Fig. 1. Substance P increased both the tonic and phasic contractile component in a dose dependent manner. But, the response of the tonic contractile component was relatively small compared to that of the phasic contractile component.

The excitatory effect of Substance P was blocked almost completely by a Substance P antagonist ($10^{-6}$M) (D-Pro[2], D-Phe[7], D-Trp[9]), but TTX ($3\times10^{-7}$ M), guanethidine ($5\times10^{-6}$ M) and atropine ($10^{-6}$ M) had no influence on the excitatory action of Substance P (Fig. 2). Whereas the stimulating effects of Substance P were potentiated by the addition of phosphoramidone, which is known as a peptidase inhibitor (Fig. 3).

### Effects of Substance P on the electrical slow waves of the antral circular muscle

The isometric tension and electrical activities of the antral circular muscle strip were simultaneously recorded using an intracellular

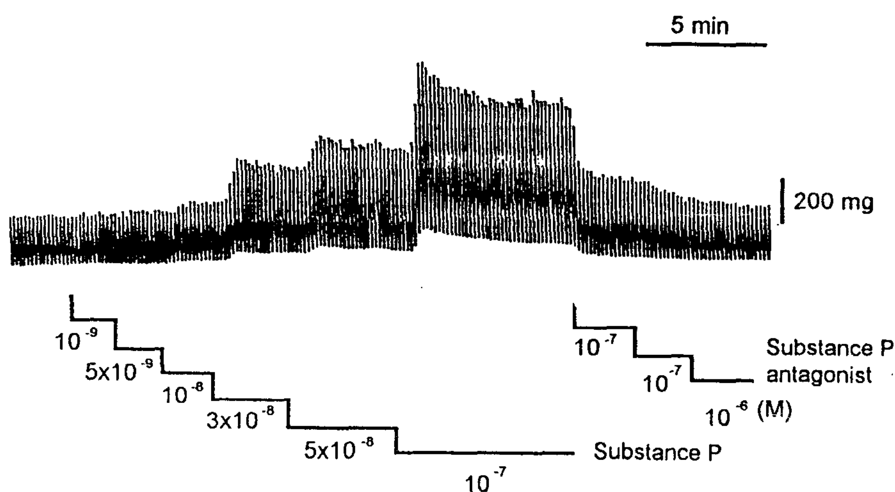


*Fig. 1. The effect of Substance P on the spontaneous contractions recorded from antral circular muscle strip of guinea-pig stomach.*

*Substance P was administered cumulatively. The excitatory effects appeared at a concentration of $10^{-9}$ M. Note that the increased amplitude of phasic contraction occurs in a dose-dependent manner, while that of tonic contraction showed a initial increase followed by a subsequent gradual decrease. This effect was bolcked by the application of the Substance P antagonist (D-Pro[2], D-Phe[7], D-Trp[9]).*

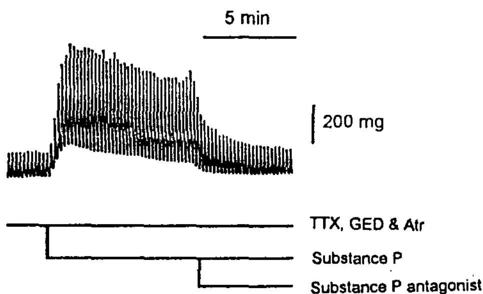


*Fig. 2. The effect of Substance P on the spontaneous contractions after pretreatment with 3 neurotransmitter blockers (TTX, Atr and GED) recorded from the antral circular muscle strip of guinea-pig stomach. Substance P-induced contractions were no changed during pretreatment with 3 neurotransmitter blockers (TTX(tetrodotoxin $3\times10^{-7}$M), GED (guanethidine $5\times10^{-6}$M) Atr (atropine $10^{-6}$ M)*

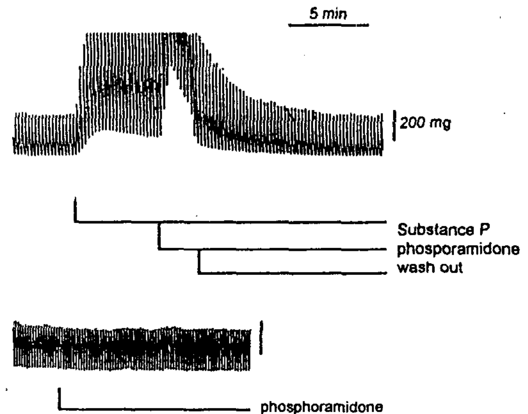


*Fig. 3. · The effect of phosphoramidone(peptidase inhibitor) on the Substance P-induced contractions recorded from the antral circular muscle strip of guinea-pig stomach. Substance P-induced contractions were potentiated by the application of phosphoramidone ($5\times10^{-7}$ M, upper trace). However, phosphoramidone itself has no direct effect on the spontaneous contractions (lower trace).*

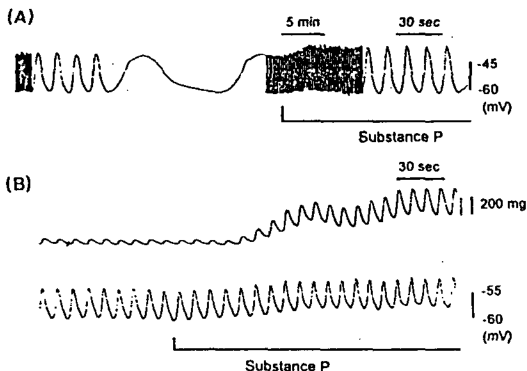


*Fig. 4. The effect of Substance P on the slow waves and spontaneous contractions recorded from the antral circular muscle of guinea-pig stomach using intracellular recording method. Note that Substance P produced depolarization of membrane and increased the amplitude of slow waves, but the frequency was not changed (A & B). At the same time spontaneous contractions were increased (B).*

microelectrode technique. The antral circular muscle of guinea-pig stomach exhibited very regular electrical slow waves. Their frequency was about 4-5/min, their membrane potential was about -60~-65 mV, and the amplitude of slow waves was 15-30 mV. Substance P produced depolarization of membrane potential and increased the amplitude of slow waves. But, the frequency was not affected. At the same time tonic and phasic contractions were increased (Fig. 4).

**Effects of Substance P on the voltage-dependent $Ca^{2+}$ currents of antral circular myocyte**

For the isolation of the inward $Ca^{2+}$ currents, we used cesium-aspartate solution in the pipette which contained TEA (tetraethylammoniumm acid), which is known as a blocking agent of outward currents. Depolarizing voltage steps from a holding potential of -60 mV to various level elicited inward currents. The inward currents proved to be $Ca^{2+}$ currents, because the currents were completely blocked by removal of $Ca^{2+}$ in the bath solution, or by the application of nifedipine or verapamil. Also, the amplitudes

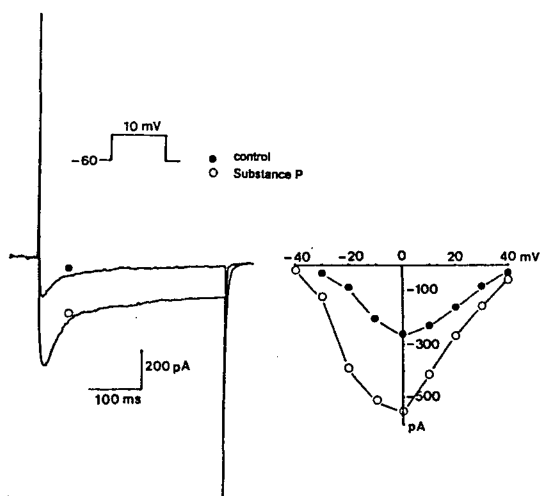


*Fig. 5. The effect of Substance P on the $Ca^{2+}$ currents in the isolated single antral myocyte. (A) shows the enhancing effect of Substance P on the $Ca^{2+}$ current by depolarization from the holding potential of -60 mV to 10 mV for 350 ms duration with a Cs-aspartate internal solution. (B) shows the current-voltage relationship for peak $Ca^{2+}$ currents before and after treatment with Substance P. The $Ca^{2+}$ currents were increased in the whole test voltage range by the application of Substance P.*

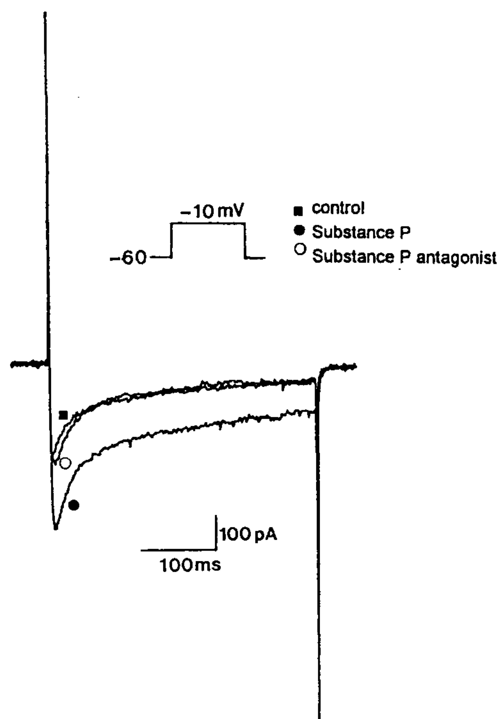


*Fig. 6. The effect of the antagonist on the Substance P-enhanced calcium currents. The inward $Ca^{2+}$ currents were evoked by the depolarizing pulse from the holding potential of -60 mV to -10 mV. By the application of antagonist ($10^{-6}$ M), Substance P-enhanced $Ca^{2+}$ currents were returned to the normal levels.*

and shapes were not changed in the presence of TTX (tetrodotoxin $10^{-7}$ M) (Rhee et al 1933). The $Ca^{2+}$ currents were activated at -30 mV and their peaks appeared at around 0 mV. Figure 5 shows the effects of Substance P on the calcium currents in the antral myocytes. (A) shows the enhancing effect of Substance P on the calcium current evoked by stimulating pulse to 0 mV from a holding potential of -60 mV for a duration of 350 ms. (B) shows the current-voltage relationship for peak calcium currents before and after the application of Substance P. Substance P increased calcium currents in the whole test voltage range. However, their activation and peak potentials were not changed. The enhancing effects on calcium currents by Substance P, were inhibited with the treatment of a Substance P antagonist and calcium current returned to normal levels (Fig. 6).

**Effects of Substance P on the outward currents of antral circular myocytes**

We recorded outward currents in the antral circular myocytes in a $K^+$-aspartate solution containing 0.5 mM EGTA in the pipette. Depolarizing voltage steps from a holding potential of -60 mV elicited inward $Ca^{2+}$ currents followed by outward $K^+$ currents of 2 types: Transient and spontaneous oscillatory outward currents and sustained outward currents. Transient and spontaneous outward currents were $Ca^{2+}$ dependent, because their currents were suppressed by the removal of

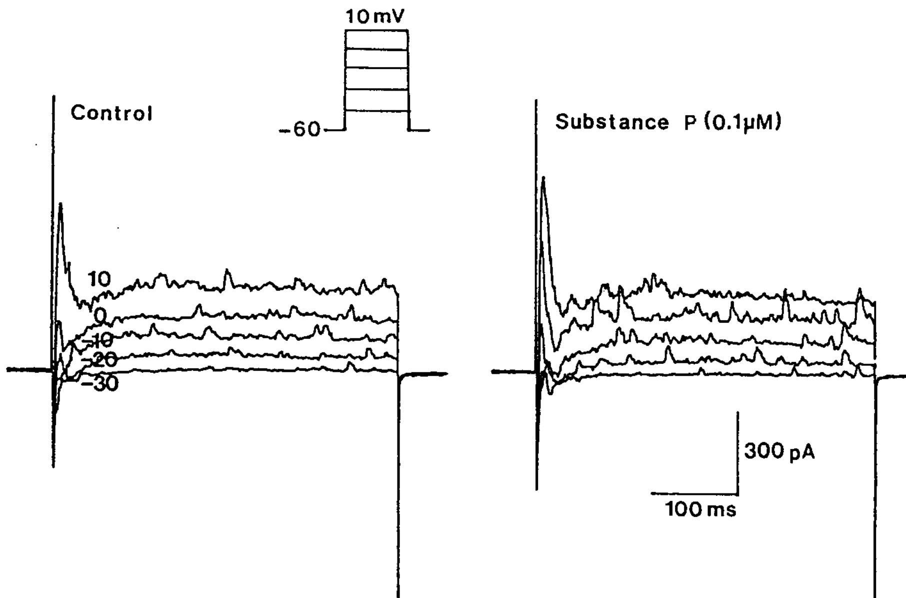


*Fig. 7. The effect of Substance P on the $K^+$ outward currents in the isolated single antral myocyte. The transient and spontaneous oscillatory $K^+$ outward currents were evoked by stimulatory pulses of various magnitudes from the holding potential of –60 mV. Application of Substance P increased the transient and spontaneous oscillatory outward currents in the whole test voltage range.*

$Ca^{2+}$ or by the application of a calcium antagonist in the bath and completely abolished by the use of a solution containing 10 mM EGTA in the pipette (Rhee et al. 1993). Figure 7 shows the effects of Substance P on the $Ca^{2+}$-dependent $K^+$ outward currents by depolarizing pulse from a holding potential of –60 mV to 10 mV with 10 mV steps of a duration of 400 ms. The application of Substance P into the bathing solution increased both transient and spontaneous oscillatory outward currents. However, sustained outward currents were not changed. These currents increased by Substance P were abolished by $10^{-7}$ M of apamin, known to be a specific blocker for $Ca^{2+}$-dependent $K^+$ channel.

## DISCUSSION

Substance P has been previously shown to contract the stomach of various mammalian species in a variety of in vitro preparations (Kazuaki & Yushito, 1982; Milenov & Golenhofen, 1983). In this experiment, Substance P increased both tonic and phasic contractions. However, tonic contractions were less sensitive than phasic contractions to Substance P. The effects of Substance P were blocked by the Substance P antagonist (Fig. 1) (D-Pro[2], D-Phe[7], D-Trp[9]). The excitatory responses of circular muscle strips elicited by the addition of Substance P were not significantly influenced by the pretreatment with atropine, guanethidine, or tetrodotoxin (Fig. 2). This suggests that responses to Substance P are due to direct action on the smooth muscle cells rather than by a neural mediated effect. Substance P responses were potentiated by $5 \times 10^{-7}$ M phosporamidone, a peptidase inhibitor, tonic contractions in particular were largely increased. Spontaneous contractions were not affected by phosphoramidone itself (Fig. 3). These effects indicate that Substance P was

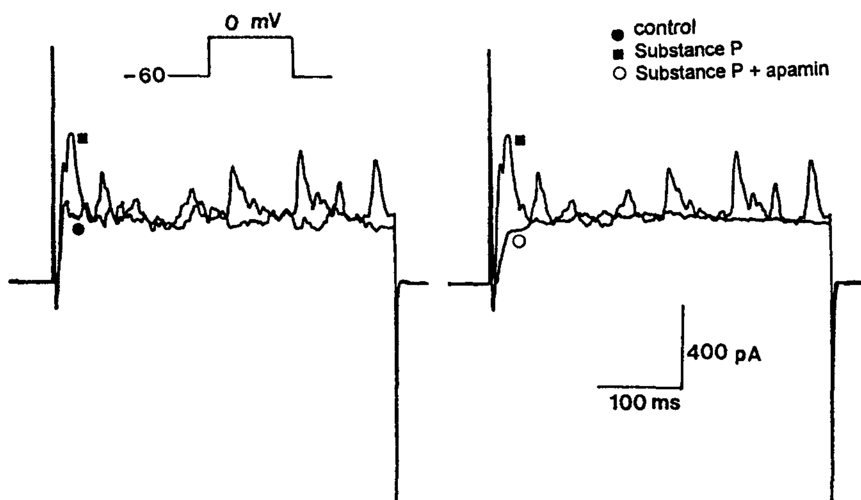


*Fig. 8. The effect of $Ca^{2+}$ activated $K^{+}$ channel bloker, apamin on the Substance P-enhanced transient and spontaneous oscillatory outward currents. Both the currents evoked by the application of a depolarizing pulse from the holding potential of –60 mV to 0 mV were increased by Substance P (A), and were abolished by $10^{-7}$ M of apamin (B).*

rapidly degraded by neutral peptidase in the circular muscle cells. Therefore, one might guess that tonic contractions were not efficiently maintained by Substance P.

Mechanical contractions of gastrointestinal smooth muscle were determined by electrical slow waves. Substance P produced depolarization of membrane potential and increased the amplitude of slow waves in muscle strips, investigated by intracellular recording (Fig. 4). But, the frequency was not changed. Slow waves are related to various ionic permeabilities (Ohba & Tomita, 1975). The precise ionic mechanisms of slow waves are not known. However, it has been suggested that electrical slow waves are related to the voltage-dependent $Ca^{2+}$ channel, because the amplitude of slow waves are decreased and abolished by $Ca^{2+}$ channel antagonists and by a $Ca^{2+}$ free in extracellular solution (Barajas & Huizinga, 1988; Ohba & Tomita, 1977). Therefore, these effects of Substance P on slow waves are associated with the changes of membrane conductance and excitability. The same effects on slow waves were reported in the intestine (Bauer & Kuriyama, 1982; Fusisawa & Ito, 1982; Sims et al, 1986). Multiple mechanisms of cellular excitation by Substance P have been reported, including influx of $Ca^{2+}$ (Koelbel et al 1989), release of $Ca^{2+}$ from intracellular stores into the cytosol (Holzer & Lippe, 1984) and a decrease in the conductance of the membrane to $K^{+}$ ions (Fujisawa & Ito, 1982; Sims et al, 1986), whereas agonist that stimulates contractions have been found to inhibit $Ca^{2+}$ activated $K^{+}$ currents (Benham and Bolton, 1986).

In order to confirm the ionic nature of the Substance P-induced changes on slow waves, we have performed whole cell patch clamp recordings in isolated gastric antral myocytes. Previous studies have demonstrated that the kinetics and pharmacological properties of voltage-dependent $Ca^{2+}$ currents in the antral myocytes are similar to other voltage-dependent $Ca^{2+}$ currents recorded in various tissues (Ohba et al, 1987; Sims, 1992). In these experiments, Substance P increased $Ca^{2+}$

currents in the whole test voltage range (Fig. 5). These results suggest that the excitatory effect of Substance P was mediated by a voltage-dependent $Ca^{2+}$ channel. The same results were reported in the mammalian colonic smooth muscle cells (Clapp et al, 1988; Mayer et al, 1990) and in amphibian gastric smooth muscle cells (Sims et al, 1988). But we cannot exclude the possibility of calcium ion release from intracellular stores, as tonic contractions were still evoked by Substance P in the pretreatment with nifedipine $10^{-5}$ M (data not shown here).

Two types of $K^+$ currents were recorded in the antral gastric myocytes; the spontaneous and transient oscillatory (STOCs), and the sustained $K^+$ outward currents (Fig. 7). STOCs were $Ca^{2+}$ dependent, because STOCs were completely blocked by removal of extracellular $Ca^{2+}$ ions and by application of nifedipine (Ohba et al, 1987; Benham & Bolton, 1986; Rhee et al, 1993). Substance P activated currents through $Ca^{2+}$ activated $K^+$ channels (Fig. 7), while having no effect on the sustained $K^+$ currents (Fig. 7). Similar results were reported in colonic smooth muscle cells (Mayer et al, 1990). This effect was blocked by apamin, a $Ca^{2+}$ activated $K^+$ channel blocker (Fig. 8).

Sims et al (1986) reported that the decrease of $K^+$ current (M-current) is related to Substance P-induced depolarization and is a mechanism of cellular excitation by Substance P in the amphibian stomach. Holzer & Petsche (1983) proposed that Substance P contracts the longitudinal smooth muscle of the guinea-pig ileum through a decrease in $K^+$ membrane permeability. However, the M-current does not exist in mammalian gastrointestinal smooth muscle cells and Substance P increased $Ca^{2+}$ activated $K^+$ currents in this experiment. Therefore, these results suggested that a mechanism of cellular excitation by a decrease of $K^+$ permeability might not be involved in depolarization of slow waves and the excitatory action of Substance P in antral myocytes of guinea-pig stomach. But we did not identify the mechanisms involved in Substance P-induced depolarization. Further investigations are required.

In conclusion, our results suggest that the excitatory inotropic effect of Substance P on the antral circular muscle of guinea-pig is mediated by a voltage-dependent $Ca^{2+}$ channel, which is activated by depolarization of the membrane potential. And, $Ca^{2+}$ activated $K^+$ currents are enhanced by the increase of intracellular $Ca^{2+}$ concentration.

## REFERENCES


Barajas-Lopez C & Huizinger J (1988) Heterogeneity in spontaneous and tetraethylammonium induced intracellular electrical activity in colonic circular muscle. *Pflügers Arch* **412,** 203-210

Bartho L & Holzer P (1985) Search for a physiological role of Substance P in gasrto-intestinal motility. *Neuroscience* **16 (1),** 1-32

Bauer V & Kuriyama H (1982) The nature of non-cholinergic, non-adrenergic transmission in longitudinal and circular muscles of the guinea-pig ileum. *J Physiol (London)* **332,** 375-391

Benham CD & Bolton TB (1986) Spontaneous transient outward currents in single visceral and vascular smooth muscle cells of the rabbit. *J Physiol (London)* **381,** 385-406

Burnstock G (1986) The changing faces of autonomic transmission. *Acta Physiol Scand* **126,** 67-91

Clapp LH, Vivaudou MB, Singer J & Walsh V (1989) Substance P like acetylcholine, augments one type of $Ca^{2+}$ current in isolated smooth muscle cells. *Pflügers Arch* **413,** 565 -567.

Demol P, Ruoff HJ, & Weihrauch (1989) Rational phamacotheraphy of gastrointestinal motility disorders. *Eur J Pediatr* **148,** 489-495

Fugisawa K, & Ito Y (1982) The effects of Substance P on smooth muscle cells and on neuro-effector transmission in the guinea-pig ileum. *Br J Pharmacol* **76,** 279-290

Hamill OP, Marty A, Neher E, Sakman B, & Sigworth FJ (1981) Improved patch clamp techniques for high resolution current record-

ings from cells and cell-free membrane patches. *Pflügers Arch* **391,** 85-100

Holzer P, & Petsche U (1983) On the mechanism of contraction and desensitization induced by Substance P in the intestinal muscle of the Guinea-pig. *J Physiol (London)* **342,** 549-568

Holzer P, & Lipple IT (1984) Substance P can contract the longitudinal muscle of the guinea pig small intestine by releasing intracellular calcium. *Br J Pharmacol* **82,** 259-267

Ito S, Kimura A, & Ohga A (1988) Developmental of non-cholinergic,non-adrenergic excitatory and inhibitory responses to intramural nerve stimulation in rat stomach. *Br J Pharmacol* **93,** 684-692

Koelbel CBM, Mayer EA, Reeve JR, Snape WZ, Patel A & Ho FJ (1989) Involvement of Substance P in noncholinergic excitation of rabbit colonic muscle. *Am J Physiol* **256,** G246-G253

Komori K, & Suzuki H (1986) Distribution and properties of excitatory and inhibitory junction potentials in circular muscle of the guinea-pig stomach. *J Physiol (London)* **370,** 339-355

Kwok YN & McIntoch CHS (1990) Release of Substance P-like immunoreactivity from the vasculary perfused rat stomach. *Eur J Pharmacol* **180,** 201-207

Mayer EA, Loo DDF, Snape WJ & Sache G (1990) The activation of calcium and calcium-activated potassium channels in mammalian colonic smooth muscle by Substance P. *J Physiol (London)* **420,** 47-71

Milenov K, & Golenhofen K (1983) Differentiated contractile responses of gastric smooth muscle to Substance P. *Pflügers Arch* **397,** 29-34

Ohba M, Sakamoto Y & Tomit T (1975) The slow wave in the circular muscle of the guinea-pig stomach. *J Physiol (London)* **249,** 211-239

Ohba M, Sakamoto Y & Tomita T (1977) Effcts of sodium, potassium and calcium ions on the slow wave in the circular muscle of the guinea-pig stomach. *J Physiol (London)* **267,** 167-180

Ohba Y, Kitamura K & Kuriyama H (1987) Cellular calcium regulates outward currents in rabbit intestinal smooth muscle cells. *Am J Physiol* **252,** C401-C410

Rhee SH & Kim (1987) Effects Ca and Ca antagonists on the spontaneous contractions and electrical activities of guinea-pig stomach. *Kor J Physiol* **21,** 241-257

Rhee PL, Lee SJ, Kim SJ, So I, Hwang SI & Kim KW (1993) Effects of dopamine on the $Ca^{2+}$ dependent $K^{+}$ currents in isolated single gastric myocytes of guinea-pig. *Kor J Physiol* **27 (2),** 139-150

Shirakawa JM, Nakanish T, Taniyama K, Kamidono S & Tanaka C (1989) Regulation of the Substance P-induced contraction via the release of acetylcholine and $\gamma$-aminobutyric acid in the guinea-pig urinary bladder. *Br J Pharmacol* **98,** 437-444

Sims, SM, Walsh JR & Singer J (1986) Substance P and acetylcholine both suppress the same $K^{+}$ current in dissociated smooth muscle cells. *Am J Physiol* **251,** C580-C587

Sims SM (1992) Calcium and potassium currents in canine gastric smooth muscle cells. *Am J Physiol* **262,** G859-G867

Tanaka DT & Grunstein MM (1986) Effect of Substance P on neurally mediated contraction of rabbit airway smooth muscle. *J Appl Physiol* **60(2),** 458-463

Uvans-Wallensten (1978) Release of Substance P-like immunoreactivity into the antral lumen of cats. *Acta Physiol Scand* **104,** 464-468