Enhancement by KW-5092, a Novel Gastroprokinetic Agent, of the Release of Acetylcholine from Enteric Neurons in the Guinea Pig Ileum

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ABSTRACT—KW-5092 ({1-[2-[[[5-(piperidinomethyl)-2-furanyl]methyl]amino]ethyl]-2-imidazolidinylidene} propanedinitrile fumarate) is a novel gastroprokinetic agent with acetylcholinesterase (AChE) inhibitory activity. The present study examined the effects of KW-5092 on intestinal contraction and on acetylcholine (ACh) release in the isolated longitudinal muscle-myenteric plexus preparation of guinea pig ileum. In the electrically stimulated preparation, KW-5092 enhanced the contraction at 10^{-9} M to 3×10^{-6} M and potentiated the ACh release at 10^{-8} M to 3×10^{-6} M. In the unstimulated preparation, KW-5092 at 10^{-8} M to 10^{-4} M evoked the contraction and ACh release. Both the contraction and the ACh release by KW-5092 were abolished by tetrodotoxin (10^{-7} M) or removal of external Ca^{2+}, and the evoked contraction was abolished by atropine (10^{-7} M). The ACh release by KW-5092 was not affected by hexamethonium (3×10^{-5} M), suggesting that the nicotinic receptor is not involved in the ACh release. Neostigmine, whose AChE inhibitory activity is equipotent to that of KW-5092, did not evoke ACh release even at 3×10^{-6} M, indicating that the ACh release by KW-5092 is not due to its AChE inhibitory activity. The present results suggest that KW-5092 evokes ACh release by stimulating a cholinergic pathway and that the ACh release by KW-5092 may contribute to its gastroprokinetic effects.

Keywords: KW-5092, Ileum (guinea pig), Acetylcholine release, Myenteric plexus

Dysfunctions of gastrointestinal motility entailed in gastroparesis, gastric stasis, postoperative ileus or gastroesophageal reflux disease have been treated effectively with gastroprokinetic agents, including domperidone (1), metoclopramide (2) and cisapride (3). These agents are known to exert their gastroprokinetic effects via different mechanisms of action. Domperidone enhances gastroduodenal coordination via blockade of a specific dopamine receptor (1). Metoclopramide exerts its gastroprokinetic effects via an antidopaminergic property and/or an increase in ACh release from postganglionic nerve endings (2). On the other hand, cisapride enhances ACh release without affecting dopamine receptors (3).

KW-5092 ({1-[2-[[[5-(piperidinomethyl)-2-furanyl]methyl]amino]ethyl]-2-imidazolidinylidene} propanedinitrile fumarate) is a newly synthesized gastroprokinetic agent, which enhances gastrointestinal motility in anesthetized rabbits (4). In the in vitro study, KW-5092 inhibits the activity of acetylcholinesterase (AChE) derived from rat brain, the inhibitory activity being equipotent to that of neostigmine (4), suggesting that KW-5092 stimulates gastrointestinal motility through AChE inhibition. Prior to the present study, however, studies have not been performed to determine if KW-5092 stimulates ACh release from the gut. In the present study, we investigated the effects of KW-5092 on mechanical activity and the release of ACh in the isolated longitudinal muscle-myenteric plexus preparation of guinea pig ileum.

MATERIALS AND METHODS

Drugs

[^{3}H]Choline (3148.7 GBq/mmol) was purchased from New England Nuclear (Boston, MA, USA). KW-5092 was synthesized in our laboratories. The following materials were purchased from the indicated sources: Atropine sulfate and sodium tetraphenylboron (Nacalai Tesque, Inc., Kyoto); tetrodotoxin (TTX), neostigmine methyl sul-

fate and choline kinase (Sigma Chemical Co., St. Louis, MO, USA); ethylene glycol bis(2-aminoethylether)-N,N, N',N'-tetraacetic acid (EGTA) (Kanto Chemical Co., Inc., Tokyo); hemicholinium-3 (Aldrich Chemical Company, Inc., Milwaukee, WI, USA); Soluene-350 (Packard, Downers Grove, IL, USA); nicotine and Scintisol® AL-1 (Wako Pure Chemical Industries, Osaka); hexamethonium dichloride (Research Biochemicals, Inc., Natick, MA, USA); adenosine-5'-triphosphate (ATP) (Funakoshi Co., Ltd., Tokyo); and Triton X-100 (Yoneyama Yakuhin Co., Ltd., Osaka). The test drugs were dissolved in the Tyrode solution (136 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl$_2$, 0.4 mM NaH$_2$PO$_4$, 5.6 mM glucose, 11.9 mM NaHCO$_3$, 1.8 mM CaCl$_2$, pH 7.4) for the mechanical experiment and in Tyrode solution containing 10^{-5} M hemicholinium-3 for the [³H]ACh release experiment. For the experiment on the removal of external Ca^{2+}, the solution was prepared so that it did not contain CaCl$_2$ but contained 10^{-3} M EGTA.

Preparation of longitudinal muscle-myenteric plexus (LM-MP)

Male Hartley guinea pigs weighing 250 to 450 g (Japan SLC, Inc., Hamamatsu) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and sacrificed. The LM-MP preparation was prepared according to the reported procedure (5). The ileum was immediately excised, and a strip about 3 cm in length was prepared from the ileum 10 cm proximal to the ileocecal sphincter.

Recording of mechanical activity of LM-MP preparation

Mechanical experiments were carried out with a slight modification of the reported procedure (6). The preparation was suspended vertically in the organ bath (10 ml) and superfused at a flow rate of 1.2 ml/min with Tyrode solution. The solution was continuously bubbled with a gas mixture (95% O$_2$+5% CO$_2$) and maintained at 37°C. The resting tension of 0.5 g was applied and kept constant by readjustment during the equilibration period. Mechanical activity was recorded on a multichannel polygraph by means of an isometric transducer (TB-611T; Nihon Kohden, Tokyo).

To determine the effects of KW-5092 on contraction of the electrically stimulated preparation, two parallel platinum electrodes were used to stimulate intramural nerves of the preparation positioned between those two electrodes. Each preparation was subjected to repeated electrical transmural stimulation (ES) for 2 min until a reproducible response was obtained. The parameters of ES were: 1 msec pulse duration, 15 V intensity and a frequency of 0.5 Hz. Each preparation was then subjected to ES for 2 min in the presence of KW-5092. The increase by KW-5092 of the ES-evoked contraction was represented as a percent increase over the ES-evoked contraction in

the absence of KW-5092.

To determine the effects of KW-5092 on contraction of the unstimulated preparation, each preparation was subjected to repeated treatment with 10^{-4} M KW-5092 for 1 min until a reproducible response was obtained. Each preparation was then singly treated with KW-5092 at 10^{-8} M to 10^{-4} M for 1 min. The amplitude of the KW-5092-evoked contraction of the unstimulated preparation was represented as a percentage of that induced by 10^{-4} M KW-5092.

Measurement of [³H]ACh release

Release experiments were carried out with a modification of the reported procedure (7). The LM-MP preparation was incubated with 5.5×10^{-8} M [³H]choline for 60 min in warmed Tyrode solution (37°C), equilibrated with a gas mixture (95% O$_2$ + 5% CO$_2$). After the preparation was washed in the fresh Tyrode solution (37°C, bubbled with the gas mixture) for 30 min, it was suspended vertically in the organ bath (10 ml) and superfused at 1.2 ml/min with the Tyrode solution containing 10^{-5} M hemicholinium-3 to prevent the uptake of choline formed from ACh. The experiment was started 30 min after the spontaneous release of tritium had reached a plateau level.

To determine the effects of KW-5092 on the release of [³H]ACh from the electrically stimulated preparation, 2 min of ES was applied successively five times to the preparation at 30-min intervals. The parameters of ES were: 1 msec pulse duration, 15 V intensity and a frequency of 0.5 Hz. The superfusate was collected every 1 min. The ES-enhanced release of [³H]ACh markedly declined from the first to the second stimulation period (by about 30%); however, there were no significant differences in the [³H]ACh release at the second to the fifth stimulations. Therefore, the release of [³H]ACh enhanced by the first stimulation was discarded, and the release enhanced by the second stimulation was used as the control. The effects of KW-5092 were determined by using the release of [³H]ACh at the third, fourth and fifth electrical stimulations.

To determine the effects of KW-5092 on the release of [³H]ACh from the unstimulated preparation, KW-5092 was applied for 1 min successively five times to the preparation at 30-min intervals. The 10^{-4} M KW-5092-evoked release of [³H]ACh markedly declined from the first to the second treatment period (by about 30%); however, there were no significant differences in the [³H]ACh release at the second to the fifth treatments. Therefore, the release of [³H]ACh evoked by the first treatment was discarded, and the release evoked by the second to the fifth treatments were used to determine the effects of KW-5092 on the release of [³H]ACh. The superfusate was collected every 1 min. The radioactivity of the superfusates and

that of the tissue dissolved in Soluene-350 at the end of release experiment were counted in a liquid scintillation counter (Model 4530; Packard, Downers Grove, IL, USA).

Extraction and separation of [^{3}H]choline and [^{3}H]ACh were carried out with a modification of the reported procedure (8). The superfusate (0.9 ml) was collected in a 10-ml assay vial and 0.6 ml of 200 mM Tris-HCl buffer, pH 8.1, containing 30 mM $MgCl_2$, 10 mM ATP and 0.006 units choline kinase was added. After a 1-hr incubation at room temperature to completely phosphorylate any residual choline, 5.4 ml of Scintisol$^®$ AL-1 and 0.6 ml isoamyl alcohol containing 18 mg of sodium tetraphenylboron were added. The vial was capped and shaken, and the two phases were allowed to separate for at least 30 min. [^{3}H]-ACh was selectively extracted into the scintillation fluid and was counted. Phosphoryl-[^{3}H]choline in the aqueous phase was then dissolved in the scintillation fluid with 6 ml of Triton X-100, and the total radioactivity was counted.

Calculation of [^{3}H]ACh release

The release of tritium was represented as the fractional rate, which was obtained according to the following formula:

Fractional rate = (Amount of tritium in the
   superfusate) / (Amount of tritium in the tissue)

The amount of tritium in the tissue at each period was calculated by adding the sum total amount of tritium in the superfusates after the period to the amount of tritium in the tissue at the end of the experiment. From each of the release curves obtained by plotting the fractionl rate of tritium against time, the percentage of increase of the tritium release evoked by ES and/or KW-5092 was calculated according to the following formula:

Percentage of increase of the tritium release
   = (Amount of tritium in the superfusate at the peak
   release) / (Average of the amounts of tritium in the
   superfusates of 4 fractions before the stimulation)
   × 100

Statistical analyses

All data are represented as means±S.E.M. Statistical significance was estimated by the paired t-test. A P-value of less than 0.05 was considered statistically significant.

RESULTS

Effects of KW-5092 on contraction and release of ACh in the electrically stimulated preparation

ES (1 msec pulse duration, 15 V intensity and a frequency of 0.5 Hz for 2 min) produced contractions of LM-

MP preparations, with the amplitude of 0.44±0.054 g (mean±S.E.M., n=6). KW-5092 at 10^{-9} M to $3 × 10^{-6}$ M enhanced the contraction evoked by ES in a concentration-dependent manner, with the EC_{50} value of $2.6 × 10^{-8}$ M (Fig. 1A).

The spontaneous release of [^{3}H]ACh from LM-MP preparations preloaded with [^{3}H]choline reached a plateau level after 30 min of superfusion, with the fractional rate of 0.11±0.0055% per minute (mean±S.E.M., n=66). ES enhanced the spontaneous release of [^{3}H]ACh by 50±5.8% (mean±S.E.M., n=24). The ES-enhanced release of [^{3}H]ACh was potentiated by KW-5092 at 10^{-8} M to $3 × 10^{-6}$ M, with the EC_{50} value of $4.9 × 10^{-8}$ M (Fig. 1B).
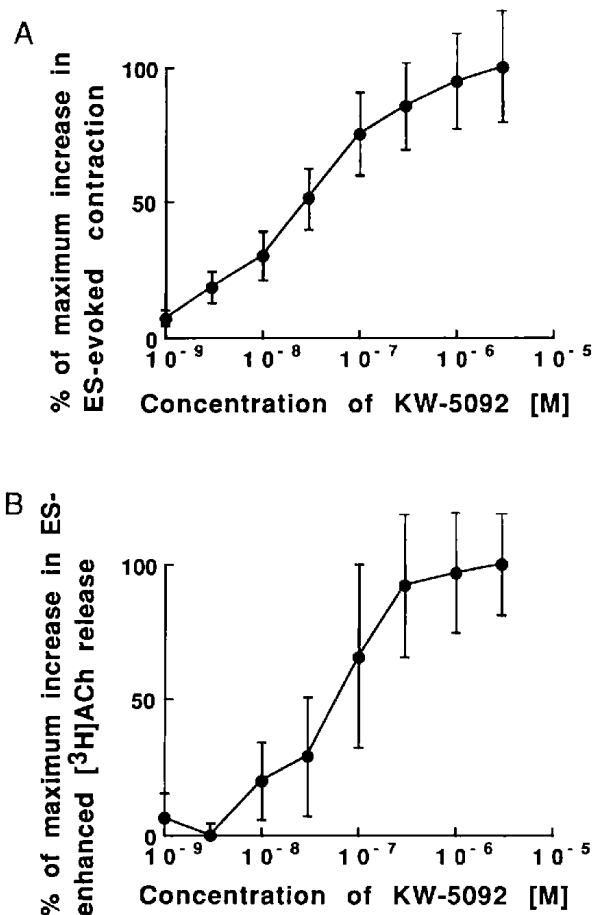

Fig. 1. Effects of KW-5092 on the contraction and the release of [^{3}H]ACh in the electrically (0.5 Hz) stimulated longitudinal muscle preparation of guinea pig ileum. A: the concentration-response curve for the KW-5092-induced augmentation of the contraction. B: the concentration-response curve for the KW-5092-induced augmentation of the electrically enhanced release of [^{3}H]ACh. Each point represents the mean±S.E.M. of 6 (A) or 8 (B) experiments.

Effects of KW-5092 on contraction and release of ACh in the unstimulated preparation

KW-5092 at 10^{-8} M to 10^{-4} M evoked contractions of LM-MP preparations in a concentration-dependent manner, with the EC_{50} value of 4.7×10^{-7} M (Fig. 2A). KW-5092 at 10^{-4} M evoked contractions with the amplitude of 0.82 ± 0.076 g (mean $\pm$ S.E.M., n = 6). The contraction evoked by KW-5092 at 3×10^{-6} M was completely inhibited by atropine at 10^{-7} M, TTX at 10^{-7} M or removal of external Ca^{2+} (Fig. 3A).

KW-5092 at 10^{-8} M to 10^{-4} M evoked the spontaneous release of [^{3}H]ACh from LM-MP preparations, with the EC_{50} value of 6.9×10^{-7} M (Fig. 2B). In contrast, neostigmine at 3×10^{-6} M did not affect the release of [^{3}H]ACh (Fig. 4).

The release of [^{3}H]ACh evoked by KW-5092 at 3×10^{-6}

Fig. 2. Effects of KW-5092 on the contraction and the release of [^{3}H]ACh in the unstimulated longitudinal muscle preparation of guinea pig ileum. A: the concentration-response curve for the KW-5092-induced enhancement of the contraction. B: the concentration-response curve for the KW-5092-enhanced release of [^{3}H]ACh. Each point represents the mean $\pm$ S.E.M. of 6 experiments.

M was completely inhibited by either TTX at 10^{-7} M or removal of external Ca^{2+} (Fig. 3B). The release of [^{3}H]ACh evoked by nicotine at 3×10^{-5} M was inhibited by hexamethonium at 3×10^{-5} M (Fig. 5A), whereas that by KW-5092 at 3×10^{-6} M was not affected by hexamethonium (Fig. 5B).

Effects of KW-5092 on the release of [^{3}H]ACh and [^{3}H]choline from the unstimulated preparation

Table 1 shows the effects of KW-5092 at 3×10^{-6} M on the release of [^{3}H]ACh and [^{3}H]choline from the unstimulated preparation. KW-5092 significantly increased the release of [^{3}H]ACh (P < 0.01), whereas it did not affect the release of [^{3}H]choline. Thus, it was confirmed that the tritium release induced by KW-5092 solely reflects the release of ACh from the cholinergic neuron.

DISCUSSION

In the previous study examining the AChE inhibitory activity in the enzyme derived from rat brain, the IC_{50} values of KW-5092 and neostigmine were 30 nM and 22 nM, respectively (4). Thus, KW-5092 is a potent AChE inhibitor, whose inhibitory activity is equipotent to that of neostigmine. In the in vivo study in anesthetized rabbits, intravenous administration of KW-5092 rapidly stimulated the motor activity of both the gastric antrum and the descending colon in a dose-dependent manner (1–10 mg/kg) (4). These results suggested that the AChE inhibitory activity of KW-5092 may contribute to its gastroprokinetic effects. On the other hand, the present study demonstrated that KW-5092 stimulates ACh release from the electrically stimulated and the unstimulated LM-MP preparations, suggesting that the action of stimulating ACh release is also involved in the gastroprokinetic effect of this drug.

The present results indicated that KW-5092 at the gastroprokinetic concentrations enhanced the contraction in the electrically stimulated preparation and evoked the con-

Table 1. Effects of KW-5092 on the release of [^{3}H]ACh and [^{3}H]-choline from the longitudinal muscle-myenteric plexus preparation of guinea pig ileum

Experimental condition	n	Radioactivity in the superfusate collected for 1 min		
		total (Bq/g tissue)	[^{3}H]ACh (Bq/g tissue)	[^{3}H]choline (Bq/g tissue)
Resting	6	220 ± 14	130 ± 6.9	89 ± 14
+KW-5092 (3×10^{-6} M)	6	$310 \pm 16^{**}$	$220 \pm 11^{**}$	85 ± 14

Values are presented as the mean $\pm$ S.E.M. of 6 experiments. **P < 0.01, compared with the value in the resting group (paired t-test).
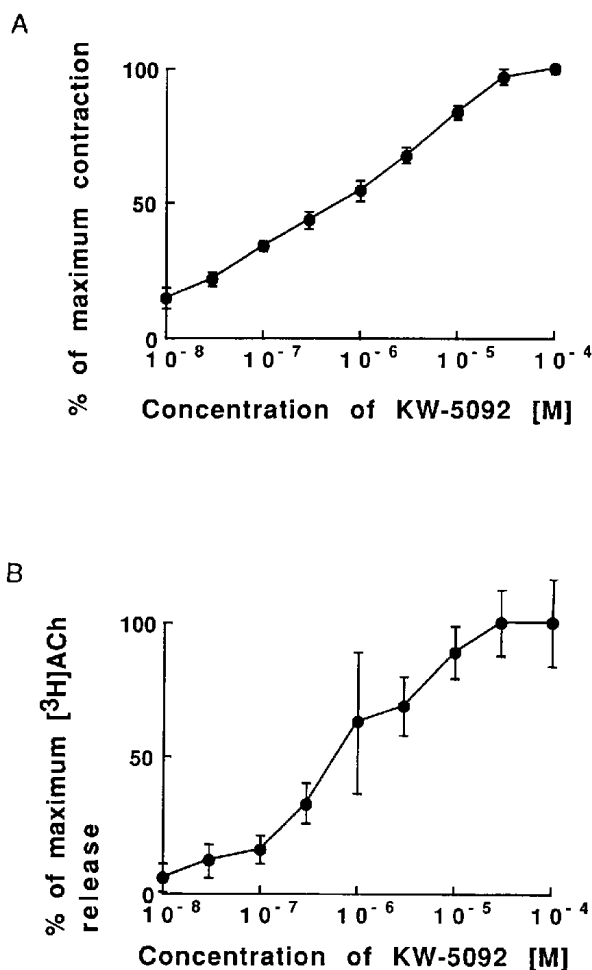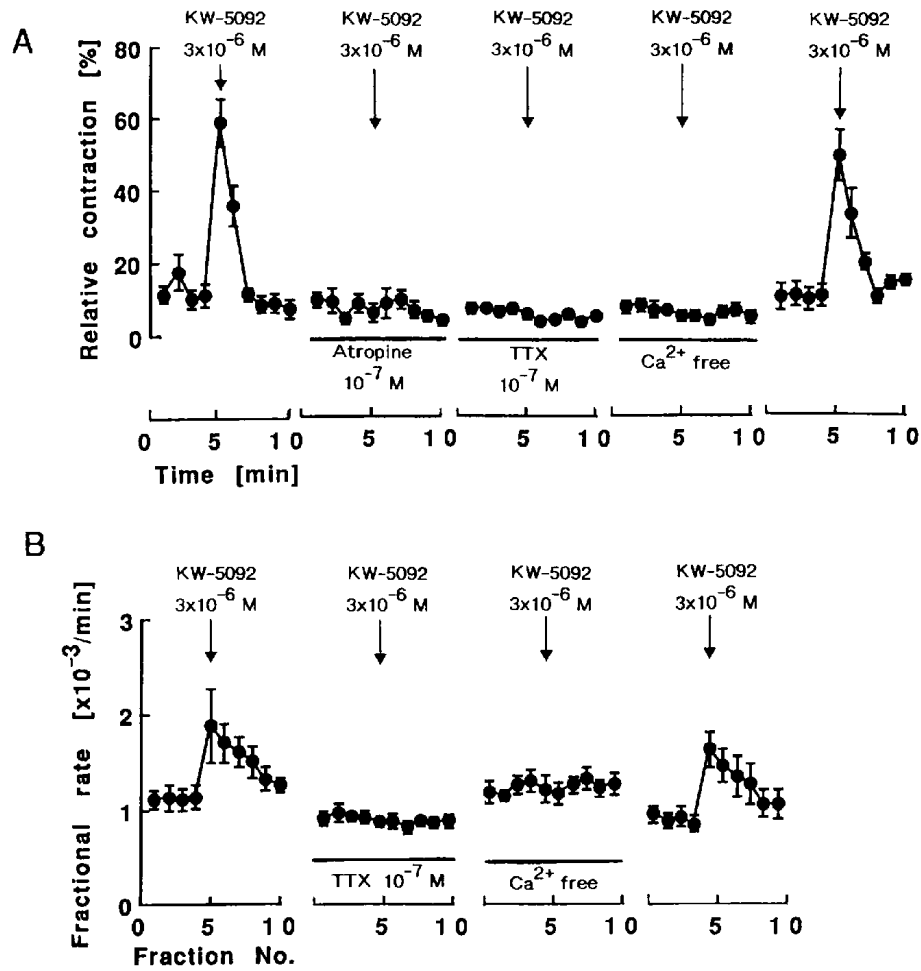
Fig. 3. Effects of atropine, TTX and removal of external Ca^{2-} on the KW-5092-evoked contraction and the release of [^{3}H]ACh in the longitudinal muscle preparation of guinea pig ileum. A: Effects on the KW-5092-evoked contraction. B: Effects on the KW-5092 release of [^{3}H]ACh. Each point represents the mean±S.E.M. of 6 experiments.

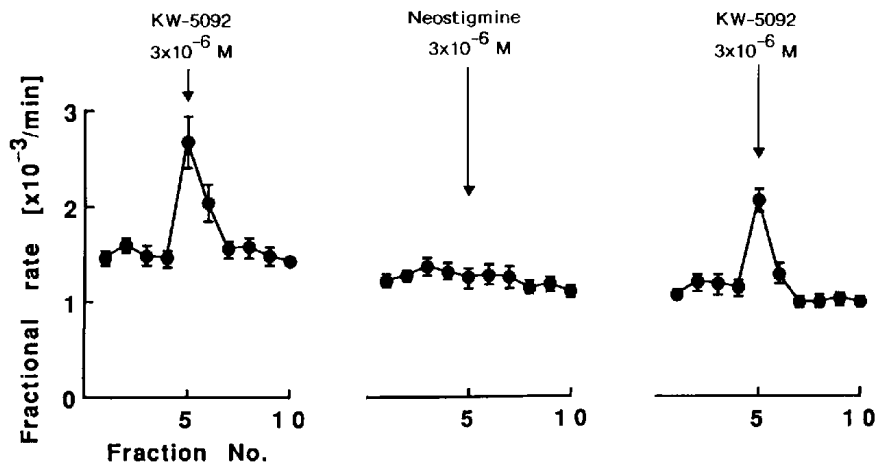**Fig. 4.** Effect of neostigmine on the release of [^{3}H]ACh from the longitudinal muscle preparation of the guinea pig ileum. Each point represents the mean±S.E.M. of 6 experiments.
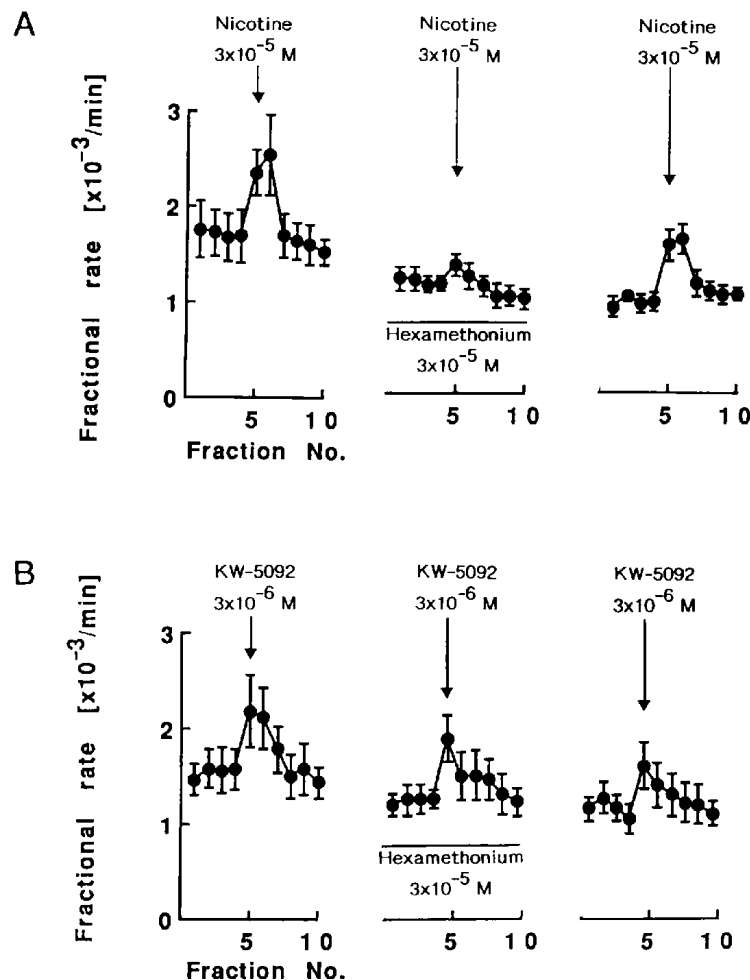
N. Kishibayashi, A. Ishii & A. Karasawa

Fig. 5. Effects of hexamethonium on the nicotine- and KW-5092-enhanced release of [³H]ACh from the longitudinal muscle preparation of guinea pig ileum. A: Effect on the nicotine-enhanced release of [³H]ACh. B: Effect on the KW-5092-enhanced release of [³H]ACh. Each point represents the mean±S.E.M. of 6 experiments.

traction in the unstimulated preparation. The contraction evoked by KW-5092 was completely inhibited by atropine, TTX or removal of external Ca^{2+}. These results suggest that KW-5092 evokes the contraction via acting on cholinergic neurons or the intrinsic neurons that transmit stimuli to cholinergic neurons.

In the present study, the release of [³H]ACh was enhanced by KW-5092 at 10^{-8} M to 3×10^{-6} M in the electrically stimulated preparation, and the release was evoked by KW-5092 at 10^{-8} M to 10^{-4} M in the unstimulated preparation. In contrast, neostigmine even at 3×10^{-6} M did not evoke the release of [³H]ACh, indicating that the enhancement of ACh release by KW-5092 cannot be ascribed to its AChE inhibitory activity. The release of [³H]ACh evoked by KW-5092 was completely inhibited by removal of external Ca^{2+}, suggesting that [³H]ACh is released from cholinergic neurons via a mechanism of exocytosis. Moreover, the KW-5092-evoked [³H]ACh

release was completely inhibited by TTX, whereas it was not affected by hexamethonium. Since TTX abolishes action potentials in the nerve by preventing the rapid entry of sodium through the depolarized cell membrane (9), and the action of TTX is limited to the nervous tissue (10), the present result suggests that KW-5092 evokes [³H]ACh release via acting, either directly or indirectly, on the cell body of the postganglionic cholinergic neuron, but not via acting on the nicotinic receptor.

KW-5092 enhanced the ES-evoked contraction with the EC_{50} value of 2.6×10^{-8} M, and it enhanced the ES-enhanced release of [³H]ACh with the EC_{50} value of 4.9×10^{-8} M. On the other hand, KW-5092 evoked contractions of the unstimulated preparation with the EC_{50} value of 4.7×10^{-7} M, and it evoked the release of [³H]ACh from the preparation with the EC_{50} value of 6.9×10^{-7} M. These results suggest that KW-5092 may act more potently on the preparation in which cholinergic neurons are ac-

tivated than on that in which cholinergic neurons are not activated. Since the AChE inhibition could lead to the increased ACh concentration in the synaptic cleft, the AChE inhibitory effect of KW-5092 might also contribute to the KW-5092-evoked contraction.

In conclusion, the present results demonstrate that KW-5092 enhances ACh release from both the electrically stimulated and the unsimulated LM-MP preparation of the guinea pig ileum. The present results suggest that KW-5092 enhances ACh release via acting on cholinergic neurons or intrinsic neurons that transmit stimuli to cholinergic neurons and that the enhancement of ACh release may contribute to its gastroprokinetic effects. Since KW-5092 possesses both AChE inhibitory and ACh releasing actions, KW-5092 could be a novel type of gastroprokinetic drug.

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