Inhibitory Effects of KW-5092, a Novel Gastroprokinetic Agent, on the Activity of Acetylcholinesterase in Guinea Pig Ileum

Nobuyuki Kishibayashi, Akio Ishii and Akira Karasawa

Department of Pharmacology, Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., 1188 Simotogari, Nagaizumi-cho, Sunto-gun, Shizuoka 411, Japan

Received June 28, 1994 Accepted September 1, 1994

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 and acetylcholine release facilitatory activity. The present study used guinea pig ileal homogenates to examine the inhibitory effects of KW-5092 on the activities of AChE and butyrylcholinesterase (BuChE). KW-5092 inhibited AChE and BuChE with the IC_{50} values of 6.8×10^{-8} M and 2.4×10^{-5} M, respectively. The IC_{50} values of neostigmine for AChE and BuChE were 3.6×10^{-8} M and 1.9×10^{-7} M, respectively. HSR-803 (*N*-[4-[2-(dimethylamino)ethoxy]benzyl]-3,4-dimethoxybenzamide hydrochloride), a gastroprokinetic agent, inhibited AChE and BuChE with the IC_{50} values of 8.6×10^{-6} M and 6.0×10^{-4} M, respectively. The AChE inhibition by KW-5092 was reversible and noncompetitive, whereas that by HSR-803 was reversible and uncompetitive. On the other hand, the AChE inhibition by neostigmine was noncompetitive when the enzyme was preincubated with this inhibitor for 2 min prior to the addition of the substrate, and it was nearly competitive when the enzyme, the inhibitor and the substrate were incubated simultaneously. The present results demonstrate that KW-5092 is a selective, reversible and noncompetitive inhibitor of AChE with different characteristics from those of neostigmine and HSR-803. The AChE inhibitory action may contribute to its gastroprokinetic effect.

Keywords: KW-5092, Neostigmine, Anticholinesterase activity, Ileum (guinea pig)

The enteric nervous system, which runs along the length of the gastrointestinal (GI) tract, plays an important role for the coordinating motor activity of the muscular layers. The dominant excitatory enteric neurotransmitter is thought to be acetylcholine (ACh). Two different enzymes that hydrolyze ACh have been described in the mammalian small intestine: acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) (1, 2). AChE is the main enzyme responsible for the inactivation of ACh, whereas the physiological function of BuChE remains unknown (3).

KW-5092 ({1-[2-[[[5-(piperidinomethyl)-2-furanyl]-methyl]amino]ethyl]-2-imidazolidinylidene} propanedinitrile fumarate) is a newly synthesized gastroprokinetic agent that dose-dependently enhances GI motility in anesthetized rabbits (4) and both conscious and anesthetized dogs (5). In conscious dogs, KW-5092 enhances the GI motility from the gastric antrum to the colon (5). In the in vitro study, KW-5092 was a potent inhibitor of AChE derived from rat brain, its inhibitory activity being equipotent to that of neostigmine (4). On the other hand, KW-5092 concentration-dependently enhanced the ACh release and the contraction in the isolated longitudinal muscle-myenteric plexus preparation of guinea pig ileum (6). These results suggest that KW-5092 stimulates GI motor activity through not only AChE inhibition but also ACh release facilitation.

In the present study, we determined the possible inhibitory effects of KW-5092 on AChE and BuChE in guinea pig ileal homogenates, and we compared them with those of neostigmine and HSR-803 (*N*-[4-[2-(dimethylamino)-ethoxy]benzyl]-3,4-dimethoxybenzamide hydrochloride), which is reported to stimulate GI motor activity through dopamine D_2-receptor blockade and AChE inhibition (7). Moreover, the characteristics of the AChE inhibition by KW-5092 was also investigated, since its inhibitory pattern has not been reported prior to the present paper.

MATERIALS AND METHODS

Drugs

KW-5092 ({1-[2-[[[5-(piperidinomethyl)-2-furanyl]methyl]amino]ethyl]-2-imidazolidinylidene} propanedinitrile fumarate) and HSR-803 (*N*-[4-[2-(dimethylamino)ethoxy]-benzyl]-3,4-dimethoxybenzamide hydrochloride) were synthesized in our laboratories. Neostigmine methyl sulfate, tetraisopropyl pyrophosphoramide (*iso*-OMPA) and 1,5-bis(4-allyldimethylammonium phenyl)pentan-3-one dibromide (BW284c51) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), acetylthiocholine (ATCh) and butyrylthiocholine (BuTCh) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka). DTNB was dissolved in 0.1 M potassium phosphate buffer (pH 7.0). Other drugs were dissolved in 0.1 M potassium phosphate buffer (pH 8.0).

Preparation of guinea pig ileal homogenates

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. Immediately thereafter, the ileum, 2 to 12 cm proximal to the ileocecal sphincter, was excised and washed with saline. For every 1 g tissue, 1 ml of 0.1 M potassium phosphate buffer (pH 8.0) was added, and the tissue was homogenated by a Polytron homogenizer (setting 5.0, 2×10 sec, Polytron® PT10-35; Kinematica GmbH Littau, Lucern, Switzerland).

Cholinesterase assay

Cholinesterase activity was measured with a slight modification of the photometric method of Ellman et al. (8) using ATCh or BuTCh as substrates. The activity was measured at 37℃ whereas in the procedure of Ellman et al. (8), it was measured at 25℃.

In the AChE assay, to 50-μl aliquots of the ileal homogenates (2 mg of protein), 2.5 ml of 0.1 M potassium phosphate buffer (pH 8.0), 0.1 ml of DTNB (final concentration, 0.3 mM), 0.1 ml of a test drug or buffer alone and 0.15 ml of *iso*-OMPA (final concentration, 1 μM), a selective inhibitor of BuChE (9), were added. The samples were preincubated at 37℃ for 2 min prior to the addition of 0.1 ml of ATCh (final concentration, 0.2 mM) to start the hydrolysis.

In the BuChE assay, to 50-μl aliquots of the ileal homogenates (2 mg of protein), 2.5 ml of 0.1 M potassium phosphate buffer (pH 8.0), 0.1 ml of DTNB (final concentration, 0.3 mM), 0.1 ml of a test drug or buffer alone and 0.15 ml of BW284c51 (final concentration, 1 μM), a selective inhibitor of AChE (10), were added. The samples were preincubated at 37℃ for 2 min prior to the addition of 0.1 ml of BuTCh (final concentration, 0.2 mM) to start the hydrolysis. The changes in optical absorbance at 412 nm were measured for 5 min by means of a spectrophotometer (U-3210; Hitachi, Ltd., Tokyo). The K_m values and the V_{max} values were determined by Lineweaver-Burk plots (11).

To determine the reversibility of the AChE inhibition by the test drugs, two 50-μl aliquots of a solution containing the ileal homogenates (2 mg of protein), preincubated with the test drug and 1 μM *iso*-OMPA, were diluted to 2.9 ml, one with 0.3 mM DTNB and the test drug and the other with 0.3 mM DTNB only. Both the samples were then assayed for enzymatic activity after the addition of 0.1 ml of ATCh (final concentration, 0.2 mM), at 0, 15, 60 and 120 min after dilution

To determine the mode of the AChE inhibition by the test drugs, aliquots of the ileal homogenates (2 mg of protein), 0.3 mM DTNB, 1 μM *iso*-OMPA and varying concentrations of the test drug were preincubated for 2 min or not preincubated, followed by adding varying concentrations of ATCh to start the enzyme hydrolysis. The changes in optical absorbance at 412 nm were measured for 5 min.

Determination of protein concentrations

According to the method of Bradford (12), the protein concentration was determined with Coomassie Brilliant Blue G (Bio-Rad Laboratories, Richmond, CA, USA) for protein binding; bovine serum albumin was used as the standard.

RESULTS

Establishment of the cholinesterase assay system

Figure 1A shows the rate of hydrolysis of ATCh by guinea pig ileal AChE as a function of protein amount. The observed rate was a linear function of protein amount up to 2 mg. The rate of hydrolysis as a function of incubation time is shown in Fig. 1B. The observed rate was a linear function of incubation time up to 5 min. Figure 1C shows the rate of hydrolysis as a function of substrate concentraction. The observed rate achieved a plateau at 0.2 mM of the substrate. The K_m value was 0.066 ± 0.0059 mM (mean±S.E.M., n=3), and the V_{max} value was 0.63 ± 0.10 (mean±S.E.M., n=3), represented by the change in absorbance at 412 nm. The K_m value was equal to the one in the AChE assay at 25℃ (0.072 ± 0.0061 mM; mean±S.E.M., n=3), and the V_{max} value was about 2 times higher than the one at 25℃ (0.38 ± 0.016; mean±S.E.M., n=3). BW284c51, a selective AChE inhibitor, at 1 μM almost completely inhibited the enzyme (data not shown).

Figure 2A shows the rate of hydrolysis of BuTCh

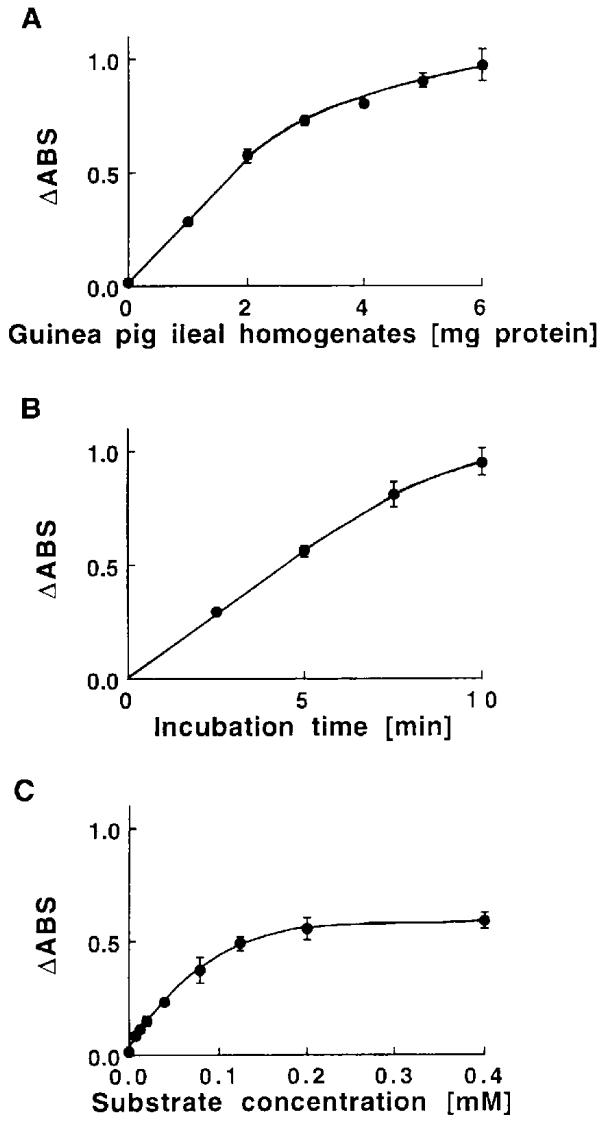

Fig. 1. Rates of hydrolysis of acetylthiocholine by guinea pig ileal acetylcholinesterase as functions of protein amount (A), incubation time (B) and substrate concentraction (C). ΔABS represents the change in absorbance at 412 nm. Each point represents the mean±S.E.M. of 3 experiments.

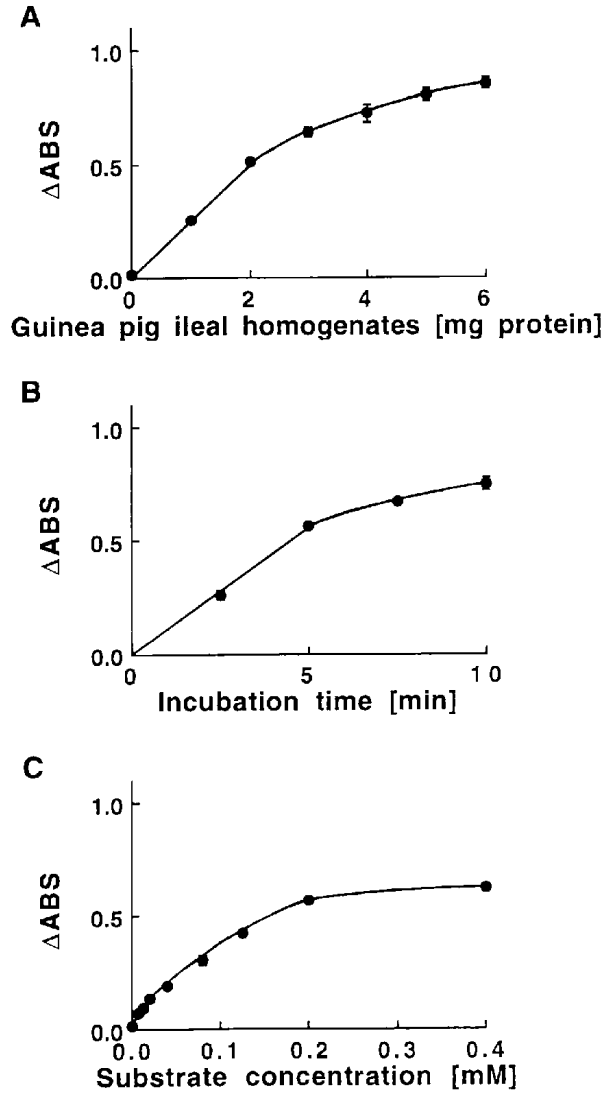

Fig. 2. Rates of hydrolysis of butyrylthiocholine by guinea pig ileal butyrylcholinesterase as functions of protein amount (A), incubation time (B) and substrate concentration (C). ΔABS represents the change in absorbance at 412 nm. Each point represents the mean±S.E.M. of 3 experiments.

by guinea pig ileal BuChE as a function of protein amount. The observed rate was a linear function of protein amount up to 2 mg. The rate of hydrolysis as a function of incubation time is shown in Fig. 2B. The observed rate was a linear function of incubation time up to 5 min. The rate of hydrolysis as a function of substrate concentraction is shown in Fig. 2C. The observed rate achieved a plateau at 0.2 mM of substrate. The K_m value was 0.086±0.0099 mM (mean±S.E.M., n=3), and the V_{max} value was 0.64±0.032 (mean±S.E.M., n=3), represented by the change in absorbance at 412 nm. *Iso*-OMPA, a selective BuChE inhibitor, at 1 μM almost completely inhibited the enzyme (data not shown).

Effects of drugs on cholinesterase

KW-5092 at 10^{-9} M to 10^{-6} M concentration-dependently inhibited the activity of guinea pig ileal AChE (Fig. 3, Table 1). Neostigmine at 10^{-9} M to 10^{-6} M and HSR-803 at 10^{-7} M to 10^{-4} M inhibited the activity in concentration-dependent manners (Fig. 3, Table 1).

KW-5092 at 3×10^{-6} M to 3×10^{-4} M concentration-dependently inhibited the activity of guinea pig ileal BuChE (Fig. 4, Table 1). Neostigmine at 3×10^{-8} M to 3×10^{-6} M and HSR-803 at 3×10^{-5} M to 3×10^{-3} M inhibited the

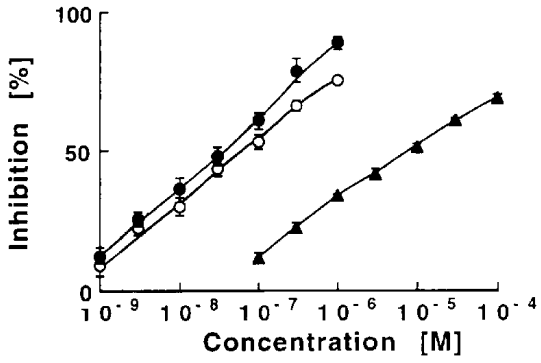

Fig. 3. Inhibitory effects of KW-5092 (○), neostigmine (●) and HSR-803 (▲) on guinea pig ileal acetylcholinesterase. Each point represents the mean±S.E.M. of 6 experiments.

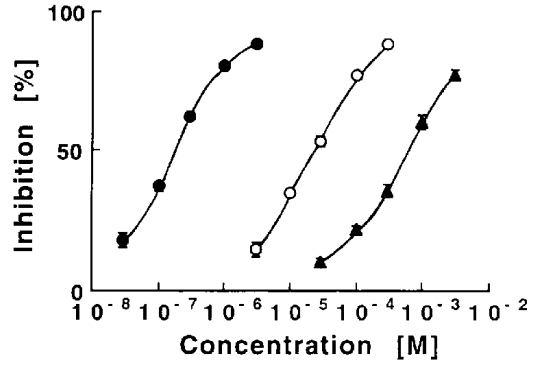

Fig. 4. Inhibitory effects of KW-5092 (○), neostigmine (●) and HSR-803 (▲) on guinea pig ileal butyrylcholinesterase. Each point represents the mean±S.E.M. of 6 experiments.

Table 1. Inhibitory effects of KW-5092, neostigmine and HSR-803 on the activities of guinea pig ileal acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE)

Drugs	IC_{50} [nM]		Ratio = $\frac{IC_{50}(BuChE)}{IC_{50}(AChE)}$
	AChE	BuChE	
KW-5092	68± 13	24,000± 1,900	350
Neostigmine	36± 7.6	190± 14	5.3
HSR-803	8,600±890	600,000±74,000	70

IC_{50} values are means±S.E.M. of 6 experiments.

activity in concentration-dependent manners (Fig. 4, Table 1).

Table 2 illustrates the AChE inhibition by KW-5092 (3×10^{-6} M), neostigmine (10^{-6} M) or HSR-803 (3×10^{-4} M) before or after a 60-fold dilution of the incubation mixture as described in the section Materials and Methods. The AChE inhibition by KW-5092 dropped from 83% to 24% just after the dilution. The AChE inhibition by HSR-803 also dropped from 79% to 23% just after the dilution. On the other hand, the AChE inhibition by neostigmine only minimally dropped from 88% to 74% even at 120 min after the dilution.

To determine the mode of the AChE inhibition by KW-5092, neostigmine or HSR-803, saturation experiments with increasing substrate concentrations were carried out. The Lineweaver-Burk plots obtained from the experimental data were linear in all the cases examined, as shown in

Table 2. Reversibility of the inhibition by KW-5092, neostigmine and HSR-803 of guinea pig ileal acetylcholinesterase (AChE) following a 60-fold dilution of drugs

Drugs	AChE inhibition (%) before dilution	Time (min) after dilution	AChE inhibition (%) after dilution	AChE inhibition (%) at diluted concentration
KW-5092	83±0.28	0	24±3.0	24±3.7
	83±0.45	15	25±3.3	
	83±0.42	60	27±0.67	
	84±1.3	120	25±3.3	
Neostigmine	88±0.38	0	87±0.91	37±1.6
	88±0.70	15	82±2.7	
	88±0.31	60	78±1.2	
	88±1.5	120	74±1.2	
HSR-803	79±0.65	0	23±3.4	23±3.7
	80±1.8	15	24±3.1	
	80±1.3	60	27±1.6	
	81±1.4	120	26±1.5	

Initial concentrations of KW-5092, neostigmine and HSR-803 were 3×10^{-6} M, 10^{-6} M and 3×10^{-4} M, respectively. The values are means±S.E.M. of 4 experiments.

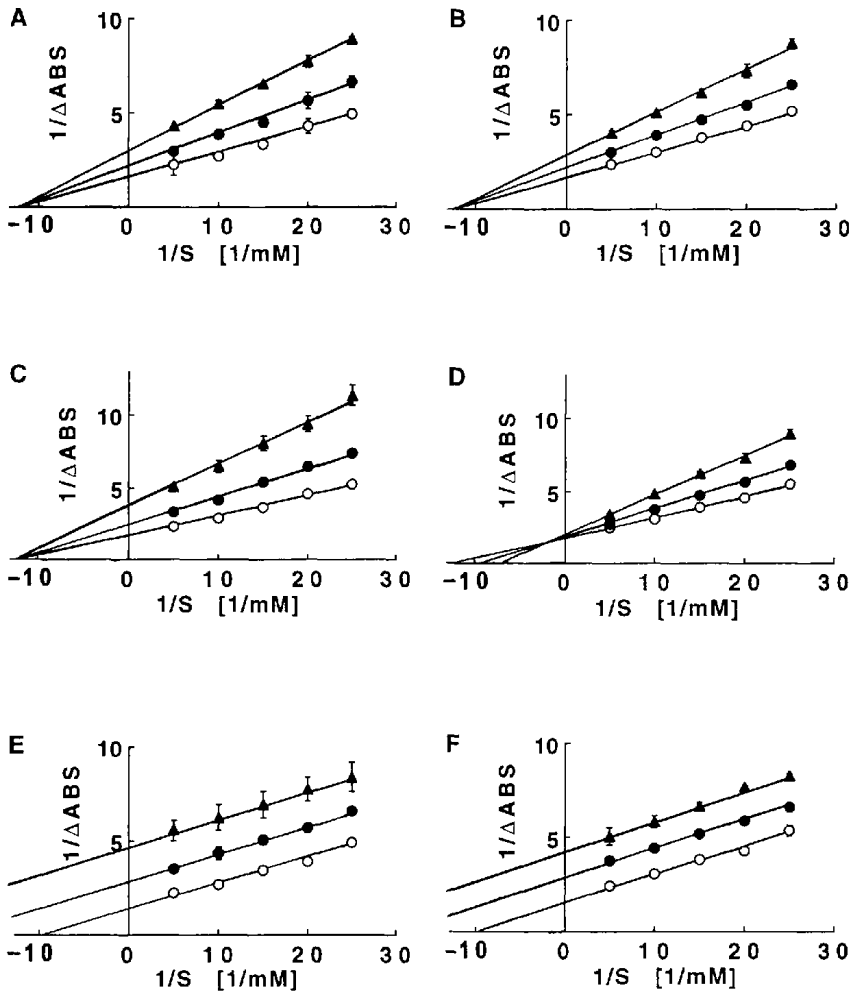

Fig. 5. Inhibition of guinea pig ileal acetylcholinesterase by KW-5092, neostigmine and HSR-803 (Lineweaver-Burk plots). The enzyme was preincubated for 2 min with KW-5092 (○: 0 nM, ●: 50 nM, ▲: 100 nM) (A), neostigmine (○: 0 nM, ●: 30 nM, ▲: 60 nM) (C) or HSR-803 (○: 0 μM, ●: 5 μM, ▲: 10 μM) (E) in the absence of acetylthiocholine, or the enzyme and the substrate (S) were simultaneously mixed with KW-5092 (○: 0 nM, ●: 50 nM, ▲: 100 nM) (B), neostigmine (○: 0 nM, ●: 30 nM, ▲: 60 nM) (D) or HSR-803 (○: 0 μM, ●: 5 μM, ▲: 10 μM) (F). Each point represents the mean±S.E.M. of 4 experiments.

Fig. 5, but they showed different patterns. The AChE inhibition by KW-5092 was noncompetitive whether or not the enzyme and the inhibitor were preincubated together for 2 min prior to the addition of the substrate (Fig. 5, A and B). The AChE inhibition by neostigmine was noncompetitive when the enzyme and the inhibitor were allowed to be preincubated for 2 min prior to the addition of the substrate, and it was nearly competitive when the enzyme, the inhibitor and the substrate were incubated simultaneously (Figs. 5, C and D). The AChE inhibition by HSR-803 was uncompetitive whether or not the enzyme and the inhibitor were preincubated together for 2 min prior to the addition of the substrate (Fig. 5, E and F).

DISCUSSION

The present study demonstrated that KW-5092 inhibits the activity of AChE derived from the guinea pig ileum. The IC_{50} value of KW-5092 was 6.8×10^{-8} M. In our previous in vitro study, KW-5092 enhanced ACh release from the longitudinal smooth muscle of the guinea pig ileum (6). In the electrically stimulated preparation, the EC_{50} value (the concentration enhancing the contraction by 50%) of KW-5092 was 4.9×10^{-8} M. Thus, KW-5092 inhibited AChE and enhanced ACh release at almost the same concentration, suggesting that both the AChE inhibition and the ACh release facilitation contribute to the stimulation by KW-5092 of the gastrointestinal motility.

KW-5092 enhances the GI motility from the gastric an-

trum to the colon in conscious dogs (5) as well as in rats (N. Kishibayashi et al., unpublished observation). In previous in vivo studies, neostigmine enhanced the propulsive motility of the colon but delayed gastric emptying in horses (13, 14). On the other hand, AS-4370, which stimulates ACh release, enhanced only the upper GI motor activity in conscious dogs (15). Moreover, our unpublished observation demonstrates that in rats, neostigmine enhances defecation without affecting gastric emptying, whereas AS-4370 enhances gastric emptying without affecting fecal output. These results suggest that the AChE inhibition mainly contributes to the enhancement of the lower GI motor activity and that the ACh release facilitation mainly contributes to the enhancement of the upper one. Thus, both the AChE inhibition and the ACh release facilitation by KW-5092 seem to be involved in the enhancement by this drug of the GI motility in a wide range from the gastric antrum to the colon.

In the present study, the IC_{50} value of KW-5092 against BuChE was markedly higher than that against AChE, indicating that the anti-cholinesterase activity of KW-5092 is selective for AChE. Similarly, the anti-cholinesterase activity of HSR-803 was also selective for AChE, although the activity was much less potent than that of KW-5092. The inhibitory action of HSR-803 on ileal AChE agrees with the previous one on stomach AChE (16). On the other hand, the IC_{50} value of neostigmine against BuChE was only slightly higher than that against AChE. The present results suggest that KW-5092 and HSR-803, in contrast to neostigmine, are selective inhibitors of AChE in guinea pig ileum. Since the control of AChE and BuChE involves different mechanisms, i.e., the distribution of AChE is tissue-specific and that of BuChE is not tissue-specific (17), KW-5092 may be more appropriate for inhibiting only the action of AChE than neostigmine.

The inhibitory action of KW-5092 on AChE was reversible, suggesting that KW-5092 immediately dissociates from the ileal AChE. Similarly, the inhibitory action of HSR-803 on this AChE was reversible, which is in accordance with the previous result on stomach AChE (18). On the other hand, the AChE inhibition by neostigmine scarcely changed even at 120 min after dilution of the enzyme mixture. The slow dissociation is assumed to be due to the carbamoylation of the enzyme by neostigmine, as was reported with physostigmine, a derivative of neostigmine (19). The present results suggest that KW-5092 and HSR-803 are reversible inhibitors of AChE, whereas the dissociation of neostigmine from AChE is very slow. Since KW-5092 dissociates from AChE immediately, the pharmacological effects of KW-5092 may correlate with the concentration of KW-5092 in the systemic circulation and thus, may be controlled easily.

The inhibitory action of KW-5092 on AChE was noncompetitive, suggesting that KW-5092 combines with the free enzyme and the enzyme-substrate complex with the same affinity. On the other hand, the inhibitory action of HSR-803 on AChE was uncompetitive, suggesting that HSR-803 combines only with the enzyme-substrate complex. The AChE inhibition by neostigmine was noncompetitive when the enzyme and the inhibitor were preincubated together, and it was nearly competitive when they were not. This observation seems to be due to the fact that, as reported with physostigmine (19), a reversible inhibitor-enzyme complex is initially formed, and then the carbamoylation of the enzyme proceeds slowly as the preincubation time is increased. Pharmacological implications for the difference in AChE inhibition among the 3 inhibitors are unclear, and further studies are required.

In conclusion, the present study demonstrates that KW-5092 is a selective, reversible and noncompetitive inhibitor of AChE in the guinea pig ileum. The mode of AChE inhibition by KW-5092 was different from those of neostigmine and HSR-803. The AChE inhibitory action may contribute to the gastroprokinetic effect of KW-5092.

Acknowledgments

We wish to thank Mr. S. Sasho for preparation of KW-5092, Mr. Y. Mimura for preparation of HSR-803 and Dr. T. Hirata for encouragement and support.

REFERENCES

1 Koelle GB: Cholinesterases of tissues and sera of rabbits. Biochem J **53**, 217–226 (1953)
2 Ambache N, Freeman M and Hobbinger F: Distribution of acetylcholinesterase and butyrylcholinesterase in the myenteric plexus and longitudinal muscle of the guinea pig intestine. Biochem Pharmacol **20**, 1123–1132 (1971)
3 Chatonnet A and Lockridge O: Comparison of butyrylcholinesterase and acetylcholinesterase. Biochem J **260**, 625–634 (1989)
4 Sasho S, Obase H, Ichikawa S, Kitazawa T, Nonaka H, Yoshizaki R, Ishii A and Shuto K: Synthesis of 2-imidazolidinylidenepropanedinitrile derivatives as stimulators of gastrointestinal motility. J Med Chem **36**, 572–579 (1993)
5 Kishibayashi N, Tomaru A, Ichikawa S, Kitazawa T, Shuto K, Ishii A and Karasawa A: Enhancement by KW-5092, a novel gastroprokinetic agent, of the gastrointestinal motor activity in dogs. Jpn J Pharmacol **65**, 131–142 (1994)
6 Kishibayashi N, Ishii A and Karasawa A: Enhancement by KW-5092, a novel gastroprokinetic agent, of the release of acetylcholine from enteric neurons in guinea pig ileum. Jpn J Pharmacol **64**, 289–295 (1994)
7 Iwanaga Y, Miyashita N, Morikawa K, Mizumoto A, Kondo Y and Itoh Z: A novel water-soluble dopamine-2 antagonist with anticholinesterase activity in gastrointestinal motor activity. Gastroenterology **99**, 401–408 (1990)
8 Ellman GL, Courtney KD, Andres V Jr and Featherstone RM: A new and rapid colorimetric determination of acetyl-

cholinesterase activity. Biochem Pharmacol **7**, 88–95 (1961)
9 Austin L and Berry WK: The selective inhibitors of cholinesterase. Biochem J **54**, 695–700 (1953)
10 Copp FC: Diacids bases. Part I. Compounds related to 1:5-diphenylpentane-pp′-bis(trialkylammonium) salts as anticholinesterases. J Chem Soc **53**, 3116–3118 (1953)
11 Lineweaver H and Burk D: The determination of enzyme dissociation constants. J Am Chem Soc **56**, 658–666 (1934)
12 Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72**, 248–254 (1976)
13 Adams SB, Lamar CH and Masty J: Motility of the distal portion of the jejunum and pelvic flexure in ponies: effects of six drugs. Am J Vet Res **45**, 795–799 (1984)
14 Adams SB and MacHarg MA: Neostigmine methylsulfate delays gastric emptying of particulate markers in horses. Am J Vet Res **46**, 2498–2499 (1985)
15 Yoshida N, Ito T, Karasawa T and Itoh Z: AS-4370, a new gastrokinetic agent, enhances upper gastrointestinal motor activity in conscious dogs. J Pharmacol Exp Ther **257**, 781–787 (1991)
16 Kimura T, Iwanaga Y, Kato K, Morikawa K, Kato H, Ito Y, Itoh Z and Kondo Y: Cholinesterase (ChE) inhibiting characteristics of HSR-803, a new gastroprokinetic agent. Jpn J Pharmacol **55**, Supp **I**, 392P (1991)
17 Edwards JA and Brimijion S: Divergent regulation of acetylcholinesterase and butyrylcholinesterase in tissues of the rat. J Neurochem **38**, 1393–1403 (1962)
18 Iwanaga Y, Miyashita N, Kato K, Morikawa K, Kato H, Ito Y, Kondo Y and Itoh Z: A novel gastroprokinetic agent HSR-803 with anti-dopamine D_2 and anti-cholinesterase activity. Eur J Pharmacol **183**, 2189 (1990)
19 Stein HH and Lewis GJ: Noncompetitive inhibition of acetylcholinesterase by eserine. Biochem Pharmacol **18**, 1679–1684 (1969)