Inhibitory Effects of KW-5092, a Novel Gastroprokinetic Agent, on the Activity of Acetylcholinesterase in 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 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)
MATERIALS AND METHODS

Drugs

KW-5092 ([1-2-[[[5-(piperidinomethyl)-2-furanyl]methyl]amino]ethyl]-2-imidazolidinylidene) propanedinitril e fumarate) and HSR-803 (N-[4-[2-(dimethylamino)ethoxy]-benzyl]-3,4-dimethoxybenzamide hydrochloride) were synthesized in our laboratories. Neostigmine methyl sul fate, 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 x 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°C whereas in the procedure of Ellman et al. (8), it was measured at 25°C.

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 0.25 ml of iso-OMPA (final concentration, 1 μM), a selective inhibitor of BuChE (9), were added. The samples were preincubated at 37°C 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), 0.05 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°C 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.

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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), 0.05 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°C 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).

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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 concentration. The observed rate achieved a plateau at 0.2 mM of the substrate. The Km 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 Km value was equal to the one in the AChE assay at 25°C (0.072±0.0061 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 Km value was equal to the one in the AChE assay at 25°C (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°C (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...
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 concentration is shown in Fig. 2C. The observed rate achieved a plateau at 0.2 mM of substrate. The $K_m$ value was $0.086 \pm 0.0099$ mM (mean±S.E.M., $n=3$), and the $V_{max}$ value was $0.64 \pm 0.032$ (mean±S.E.M., $n=3$), represented by the change in absorbance at 412 nm. Iso-OMPA, a selective BuChE inhibitor, at 1 $\mu$M 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 $3 \times 10^{-6}$ M to $3 \times 10^{-4}$ M concentration-dependently inhibited the activity of guinea pig ileal BuChE (Fig. 4, Table 1). Neostigmine at $3 \times 10^{-5}$ M to $3 \times 10^{-6}$ M and HSR-803 at $3 \times 10^{-7}$ M to $3 \times 10^{-3}$ M inhibited the
activity in concentration-dependent manners (Fig. 4, Table 1).

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

| Drugs     | IC₅₀ [nM] AChE | IC₅₀ [nM] BuChE | Ratio IC₅₀ BuChE/IC₅₀ AChE |
|-----------|---------------|-----------------|--------------------------|
| 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                       |

IC₅₀ values are means ± S.E.M. of 6 experiments.

### 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.
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 $6.8 \times 10^{-8}$ 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 $4.9 \times 10^{-8}$ 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 non-competitive, 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.

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