Characterization of Acetylcholinesterase-Inhibition by Itopride

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Received March 31, 1994 Accepted August 4, 1994

ABSTRACT—Itopride is a gastroprokinetic benzamide derivative. This agent inhibited both electric eel acetylcholinesterase (AChE) and horse serum butyrylcholinesterase (BuChE). The IC₅₀ of itopride with AChE (2.04 ± 0.27 μM) was, however, 100-fold less than that with BuChE, whereas in the case of neostigmine with AChE (11.3 ± 3.4 nM), it was 10-fold less. The recovery of AChE activity inhibited by 10⁻⁷ M neostigmine was partial, but that inhibited by upto 3 × 10⁻⁵ M itopride was complete when the reaction mixture was subjected to ultrafiltration. Double reciprocal plots of the experimental data showed that both Km and Vmax were affected by itopride, suggesting that the inhibition is a "mixed" type, although primarily being an uncompetitive one. The inhibitory effect of itopride on cholinesterase (ChE) activity in guinea pig gastrointestinal tract was much weaker than that on pure AChE. However, in the presence of a low dose of diisopropyl fluorophosphate, just enough to inhibit BuChE but not AChE, the IC₅₀s of itopride against ChE activities were found to be about 0.5 μM. In conclusion, itopride exerts reversible and a "mixed" type of inhibition preferably against AChE. The IC₅₀ of itopride for electric eel and guinea pig gastrointestinal AChE inhibition was 200 times and 50 times as large as that of neostigmine, respectively.

Keywords: Itopride, Acetylcholinesterase, Butyrylcholinesterase, Stomach (guinea pig), Acetylcholine-induced contraction

Itopride hydrochloride, N-[p-[2-(dimethylamino)ethoxy]benzyl]veratramide hydrochloride, is a newly synthesized gastroprokinetic agent with a benzamide structure. In conscious dogs, itopride stimulates gastric motility and enhances acetylcholine (ACh)-induced gastric contraction (1). In in vitro studies, itopride dose-dependently inhibits the electric eel acetylcholinesterase (AChE) activity (1). Itopride enhances ACh-induced contraction of guinea pig ileum, but not carbachol-induced contraction (2). Sakaguchi et al. reported that AChE inhibition by some newly synthesized benzamide derivatives, including itopride, is closely related to the contractile response of the ileum (3). AChE inhibitors stimulate gastrointestinal motor activity, and neostigmine has been used to treat intestinal paresis. Thus AChE inhibition by itopride may be responsible for the in vivo gastric stimulation of itopride. This study was conducted to: 1) demonstrate the inhibition properties of itopride against AChE, 2) demonstrate the inhibitory effect of itopride on crude cholinesterase (ChE) from the gastrointestinal tract and 3) examine the effects of itopride on ACh-induced contraction in guinea pig stomach at concentrations at which itopride inhibits AChE activity.

MATERIALS AND METHODS

Effect of itopride on ChE activity

Preparation of crude ChE from guinea pig gastrointestinal tract: Male Hartley guinea pigs, weighing 384–602 g, were sacrificed; and the whole stomach, a 10-cm portion of the jejunum, approximately 2 cm distal to the Treitz ligament, and a 10-cm portion of the distal colon were quickly dissected out and used. The stomach was opened along the greater curvature and the jejunum and colon, along the mesenteric border. The organs were then rinsed in ice-cold 0.32 M sucrose, and fat and mucosa were removed from the smooth muscle layer. These smooth muscle preparations were then separately weighed and well minced with scissors. The minced tissues suspended in 3 volumes of 0.32 M sucrose were homogenized twice for 20 sec each with a homogenizer (Polytron PT10-35; Kinematica, Luzern, Switzerland) set at speed level 8 and then centrifuged at 10,300 × g for 10 min at 4 °C. The supernatant fractions were recentrifuged at 164,600 × g for 30 min in an ultracentrifuge (70P-72; Hitachi, Tokyo). The final supernatant and precipitate fractions thus obtained served as crude ChE preparations, which were kept...
frozen until use. The protein content of the crude ChE was measured by the dye method (4).

**Assay of ChE activity:** ChE activity was measured at 30°C and pH 8.5 by the photometric method of Ellman et al. (5). The reaction mixture contained 2.76 ml of 0.1 M Tris-HCl buffer (pH 8.5), 0.10 ml of 0.1 M 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 0.10 ml of either itopride, neostigmine or Tris-HCl buffer (as a control). Twenty microliters of AChE (7.5 units/ml), BuChE (7.5 units/ml) or the crude ChE (2.25–7.75 mg protein/ml) was then added to the reaction mixtures containing itopride or neostigmine. In the preliminary study, inhibition by itopride was independent of preincubation time, while that by neostigmine reached a steady state after a preincubation of 20 min or more. Itopride and neostigmine containing mixtures were preincubated at 30°C for 3 and 30 min, respectively. To initiate the reaction, 20 µl of the substrate (final concentration of 5.0 x 10^{-4} M) was added to the reaction mixture. The ChE activity of crude tissue preparations was measured in the presence and absence of 1 x 10^{-4} M diisopropyl fluorophosphate (DFP). Acetylthiocholine (ATCh) was used as a specific substrate for AChE and ChE, and butyrylthiocholine (BuTCh) was used for BuChE. ATCh was also used as a substrate of crude ChE. To elucidate the type of AChE inhibition by itopride, AChE activity was measured at increasing ATCh concentrations. ChE activity was determined by measuring the change in absorbance at 412 nm for 2 min with a spectrophotometer (U-2000, Hitachi). Enzyme activity was expressed as increase in absorbance per minute.

**Reversibility of AChE inhibition by itopride, neostigmine and DFP:** Reversibility of AChE inhibition by itopride, neostigmine and DFP was assessed by repeated ultrafiltration. The sample mixtures containing 3.70 ml of 0.1 M Tris-HCl buffer, 133 µl of 10 mM DTNB, 133 µl of test material (or Tris-HCl buffer as a control) and 26.7 µl of AChE (7.5 units/ml) were preincubated for 30 min at 30°C. Each mixture was then divided into 2 equal portions. One portion was used for AChE assay by adding 26.7 µl of ATCh. The enzyme assay value was compared with that of the control mixture without inhibitors to determine the original inhibitor activity. To examine the reversibility of the AChE inhibition by drugs, we applied repeated ultrafiltration and dilution for dialysis to remove the drug from AChE in the sample mixtures. Thus, the other half of the mixture was concentrated in an ultrafiltration device (Centricon-30; Amicon, Beverly, MA, USA) by centrifugation (SCR20BA, Hitachi) at 1,000 x g for 60 min at 4°C. The concentrate was diluted with 2.0 ml Tris-HCl buffer. The ultrafiltration and dilution were repeated twice following the same protocol. The final concentrate was diluted with 2.0 ml Tris-HCl buffer containing 3.33 x 10^{-4} M DTNB, and its AChE activity was measured as described above.

**Effects of itopride and neostigmine on ACh-induced contraction in isolated guinea pig stomach**

The guinea pig stomach was opened along the greater curvature, and the mucosa was carefully removed from the muscle layer with scissors. Circular muscle preparations of the gastric body about 2 mm in width and 10 mm in length were obtained by cutting along the circular axis. Each preparation was suspended in an organ bath containing Tyrode solution (pH 7.4, 37°C) consisting of 137.9 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl2, 0.5 mM MgCl2, 1.1 mM NaH2PO4, 11.9 mM NaHCO3 and 5.6 mM glucose. The Tyrode solution was bubbled with room air. The stomach was subjected to an initial tension of 1.0 g. ACh was cumulatively added, and the tension was recorded isometrically by using a force displacement transducer (TB-612T; Nihon Kohden, Tokyo). Dose-response curves for ACh were obtained in the presence or absence of itopride or neostigmine. The contractile response was expressed as a percentage of the maximum response on the dose-response curves without itopride or neostigmine.

**Drugs**

Acetylthiocholine iodide was purchased from Nacalai Tesque (Kyoto) or Sigma Chemicals (St. Louis, MO, USA). Butyrylthiocholine iodide and neostigmine bromide were from Nacalai Tesque. Pure acetylcholinesterase (EC3.1.1.7, electric eel) and butyrylcholinesterase (EC3.1.1.8, horse serum) were purchased from Sigma Chemicals. Diisopropyl fluorophosphate and 5,5'-dithio-bis(2-nitrobenzoic acid) were obtained from Wako Pure Chemical Industries (Tokyo). Acetylcholine chloride was purchased from Daiichi Seiyaku Co., Ltd. Itopride hydrochloride was synthesized by Hokuriku Seiyaku Co., Ltd.

**Data analysis**

IC_{50}s of itopride and neostigmine were graphically determined from the 50% inhibition levels in the dose-response curves. All data are expressed as means±S.E.M. Statistical significance was examined by Student’s t-test. A difference was considered significant at P<0.05.

**RESULTS**

**Inhibition of ChE activity by itopride**

To characterize the inhibition of ChE activity by itopride, its inhibitory effect on electric eel AChE was compared with that on horse serum BuChE. Itopride inhibited AChE and BuChE activity dose-dependently...
Inhibitory action on AChE was about 100 times stronger than that on BuChE (AChE: IC$_{50}$=2.04±0.27 µM, BuChE: IC$_{50}$=252±20 µM). Neostigmine inhibited AChE (IC$_{50}$=11.3±3.4 nM) more potently than BuChE (IC$_{50}$=120±15 nM). The IC$_{50}$ ratio between AChE and BuChE for itopride was 10.5 times as large as that for neostigmine, suggesting greater selectivity of itopride than neostigmine toward AChE.

The reversibility of AChE inhibition by itopride was compared with that by neostigmine and DFP. Submaximal inhibition occurred at 3×10$^{-5}$ M for itopride, 1×10$^{-7}$ M for neostigmine and 1×10$^{-5}$ M for DFP (Table 1). Itopride-induced AChE inhibition was completely abolished by dialysis, whereas that caused by neostigmine decreased to a half; and as far as DFP is concerned, there was only a slight decrease in inhibition (Table 1). This clearly shows that itopride is a reversible AChE inhibitor.

To determine the type of AChE inhibition by itopride, saturation experiments with increasing substrate concentration were carried out. Double reciprocal plots from the experimental data were linear at all itopride concentrations (Fig. 2). AChE inhibition in the presence of increasing concentrations of itopride produced straight lines characterized by increasing slopes and intercepts on the 1/V axis, although the slope variation was very small. $K_m$ and $V_{max}$ were thus affected by itopride.

Inhibitory effects of itopride and neostigmine on crude ChE from guinea pig gastrointestinal tract

The effects of itopride and neostigmine on crude ChE activity obtained from three gastrointestinal sources, stomach, jejunum and colon, were examined. Figure 3

Table 1. Reversibility of electric eel acetylcholinesterase inhibition by itopride, neostigmine and diisopropyl fluorophosphate

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mol</th>
<th>n</th>
<th>% Activity of acetylcholinesterase before ultrafiltration</th>
<th>% Activity of acetylcholinesterase after ultrafiltration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Itopride</td>
<td>1×10$^{-5}$</td>
<td>4</td>
<td>23.4±2.7</td>
<td>102.4±3.3</td>
</tr>
<tr>
<td></td>
<td>3×10$^{-5}$</td>
<td>4</td>
<td>11.8±0.7</td>
<td>96.4±2.2</td>
</tr>
<tr>
<td>Neostigmine</td>
<td>1×10$^{-7}$</td>
<td>3</td>
<td>11.5±0.6</td>
<td>47.4±3.0</td>
</tr>
<tr>
<td>DFP</td>
<td>5×10$^{-6}$</td>
<td>3</td>
<td>33.5±0.9</td>
<td>47.2±1.4</td>
</tr>
<tr>
<td></td>
<td>1×10$^{-5}$</td>
<td>3</td>
<td>7.0±1.1</td>
<td>22.8±6.6</td>
</tr>
</tbody>
</table>

Acetylcholinesterase (AChE) was incubated with each drug for 30 min at 25°C. After incubation, each sample was divided into 2 portions; one was used for measuring AChE activity (before ultrafiltration), and the other was filtered through an ultrafiltration membrane and washed twice with Tris-HCl buffer. The latter was analyzed for AChE activity (after ultrafiltration). Each value represents the mean±S.E.M. of 3–4 experiments performed in duplicate. DFP: diisopropyl fluorophosphate.

Fig. 1. Inhibitory effects of itopride (circle) and neostigmine (triangle) on electric eel acetylcholinesterase (AChE) activity (filled symbol) and horse serum butyrylcholinesterase (BuChE) activity (open symbol). Values represent means±S.E.M. of 4 experiments performed in duplicate.

Fig. 2. Inhibition of electric eel acetylcholinesterase by itopride: double reciprocal plots. Acetylthiocholine in the range of concentrations S: 3.3×10$^{-5}$, 5.0×10$^{-5}$, 1.0×10$^{-4}$ and 2.5×10$^{-4}$ M. V = Δ absorbance/min. Buffer (○), 1.09×10$^{-6}$ M (●), 2.18×10$^{-6}$ M (▲), 4.36×10$^{-5}$ M (■) itopride. Values represent means±S.E.M. of 3 experiments performed in duplicate.
shows the inhibitory effects of itopride and neostigmine on each of the ChEs of the supernatant fractions from the three sources. Itopride inhibited the crude ChE activity in a dose-dependent manner, and the inhibitory action on the crude ChEs derived from the three organs was essentially the same (Table 2 and Fig. 3). Inhibition by itopride of crude ChE was extremely weak compared to its electric eel AChE inhibition. Dose-response curves of itopride for crude ChE shifted to the left by $1 \times 10^{-8}$ M DFP, and the IC$_{50}$ of itopride in the presence of DFP for the three organs was 300–600 times less than in the absence of DFP (Table 2 and Fig. 3). DFP inhibited BuChE activity dose-dependently (data not shown), and $1 \times 10^{-8}$ M DFP almost completely inhibited BuChE (94.4±0.9%). DFP (1×$10^{-8}$ M) could induce only a slight shift to the left in the neostigmine dose-response curve, whereas in the case of the itopride dose-response curve, DFP at the same concentration could induce a significant left shift (Table 2 and Fig. 3). This clearly shows that itopride selectively inhibits AChE in the gastrointestinal.

**Table 2. Inhibitory effects of itopride and neostigmine on crude cholinesterase from guinea pig gastrointestinal tract**

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>IC$_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>with DFP</td>
</tr>
<tr>
<td>Supernatant fraction</td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td>0.435±0.063</td>
</tr>
<tr>
<td>Jejunum</td>
<td>0.402±0.048</td>
</tr>
<tr>
<td>Colon</td>
<td>0.569±0.074</td>
</tr>
<tr>
<td>Precipitate fraction</td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td>0.530±0.161</td>
</tr>
<tr>
<td>Jejunum</td>
<td>0.566±0.099</td>
</tr>
<tr>
<td>Colon</td>
<td>0.624±0.060</td>
</tr>
</tbody>
</table>

DFP: Diisopropyl fluorophosphate. Each value represents the mean±S.E.M. of 3 experiments performed in duplicate.

**DISCUSSION**

Itopride is a newly synthesized gastroprokinetic agent that was found to stimulate gastric emptying in dogs and rats and small intestinal transit in mice (6). In conscious dogs, itopride stimulates postprandial gastric motility and enhances gastric contraction evoked by ACh infusion (1). Itopride inhibits electric eel AChE (IC$_{50}$=2.9 nM) (1) and enhances guinea pig ileum contraction evoked by ACh (2) in vitro. These results suggest that AChE inhibition is in-
Involved in the mechanism of the itopride-induced stimulation of gastric motility in conscious dogs. Thus, in the present study, we further characterized the inhibitory action of itopride on ChE activity.

AChE, also known as true-ChE, is found in cholinergic neurons and is the enzyme that terminates the action of ACh at the junctions of cholinergic nerve endings. BuChE, so-called pseudo-ChE, is present not only in neuronal elements of the central and peripheral nervous system but also in plasma, erythrocytes, liver and other organs. BuChE is able to hydrolyze ACh, but its physiological function is not yet known. Itopride dose-dependently inhibited both AChE and BuChE. The IC₅₀ of itopride for AChE inhibition was, however, 100 times lower than that for BuChE inhibition, demonstrating selective inhibition of AChE by the drug. Under the same experimental conditions, the IC₅₀ of neostigmine for the inhibition of AChE was also lower when compared to that of BuChE. However, the IC₅₀ ratio of neostigmine between AChE and BuChE was 10 times less than that with itopride. Atack et al. reported that the IC₅₀ of neostigmine for AChE inhibition was about two times less than that for BuChE inhibition (7). Therefore, the higher selectivity towards AChE inhibition seems to be a unique characteristic of itopride action. Atack et al. also reported that IC₅₀s of DFP for the inhibition of AChE and BuChE were 76 nM and 0.2 nM, respectively (7). These values are consistent with the present study that 10 nM DFP completely inhibited BuChE activity without affecting AChE.

The double reciprocal plots as shown in Fig. 2 are very similar to that reported by Galli et al. (8) on the histamine H₂-antagonist ranitidine. It was reported that the IC₅₀ of ranitidine for AChE inhibition was 3.5 × 10⁻⁶ M; and at doses of 1 and 3 mg/kg/hr, ranitidine significantly enhanced ACh-induced gastric contractions in conscious dogs (9), showing that ranitidine and itopride were equipotent in vitro and in vivo. Three classes of AChE inhibitors are known (10). Quaternary compounds such as edrophonium inhibit the enzyme reversibly by combining with the active center. Drugs such as neostigmine possessing a carbamyl ester linkage are hydrolyzed by AChE, but much more slowly than ACh. Organophosphorus inhibitors such as DFP inhibit the enzyme irreversibly by serving as true hemisubstrates. Regarding their chemical structures, itopride and ranitidine differ from the three classes of AChE inhibitors. Accordingly, itopride and ranitidine are similar in some respects. Galli et al. conclude that the ranitidine action was a "mixed" type inhibition, as the double reciprocal plots of AChE inhibition by increasing concentrations of ranitidine gave straight lines characterized by increasing slopes and intercepts on the 1/V axis, although the slope variation was very small (8). The inhibition of the "mixed" type contains characteristics common to both "competitive" and "non-competitive" inhibition and can occur when a reversible inhibitor combines with a free enzyme and the enzyme-substrate complex with different affinities. In "mixed" type inhibition, Kₘ and Vₘₐₓ are affected by the drug. Aono et al. (11) also analyzed the ranitidine action on AChE and reached the same conclusion on the type of inhibition as reported by Galli et al. (8). Similar analysis of the present data may suggest that the itopride action is primarily an uncompetitive type inhibition with only a small contribution of the competitive type interaction, especially in the higher concentration...
range.

The reversibility of AChE inhibition by itopride was compared with that of neostigmine and DFP. AChE inhibition by itopride was almost completely abolished by dia
alys.

The inhibitory action of neostigmine on AChE was not as readily reversible as that of itopride. DFP is an irreversible AChE inhibitor (12), and AChE inhibition by DFP was confirmed to be irreversible in this study also.

AChE is a complex family of molecular forms (13, 14). The most common forms are monomers, dimers and tetrarimers of the same subunit, plus an asymmetric form consisting of three tetrarimers covalently linked via di
sulfide bonds to a three-stranded collagen-like tail. Tsim et al. reported asymmetric AChE to contain a hybrid of AChE and BuChE catalytic subunits linked to a common collagen-like tail (15). The inhibitory potency of each of the various inhibitors toward AChEs differed according to the source of AChE (7). In the presence of 10 nM DFP, where BuChE activity was abolished, itopride showed selective inhibition of AChE. In isolated guinea pig stomach, itopride at $1 \times 10^{-6}$ and $1 \times 10^{-5}$ M enhanced ACh-induced contraction. This result shows that itopride enhances ACh-induced gastric contraction not through BuChE inhibition but through AChE inhibition. Adler et al. showed a competition between BuChE and AChE in the regulation of synaptically released ACh concentrations in canine trachealis muscle, thus elucidating a functional role for BuChE in this tissue (16). In rabbit duodenum, however, the increase in tone produced by DFP appears to be due to AChE inhibition by the agent rather than BuChE (17). The results of the present study and previous report (17) indicate that AChE may possibly be more important than BuChE in the gastrointestinal motor stimulation of ChE inhibitors.

It is evident from the present results that itopride exerts a selective and reversible AChE inhibition and enhances ACh-induced contraction in isolated guinea pig stomach.

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