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EFFECT OF RANITIDINE ON ILEAL MYENTERIC PLEXUS PREPARATION AND ON ACETYL- AND BUTYRYLCHOLINESTERASE

ALESSANDRO GALLI, PIERO MANTOVANI and GIANCARLO PEPEU
Department of Pharmacology, University of Florence, Viale G. B. Morgagni 65, 50134 Firenze, Italy

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Abstract—Ranitidine at concentrations from 1 μM to 0.1 mM brought about a dose-dependent potentiation of the twitch responses elicited by electrical stimulation of the ileal myenteric preparation. At higher concentrations (0.3–3 mM) ranitidine also caused irregular slow contractions of the unstimulated ileal preparation which were potentiated by eserine and blocked by atropine and tetrodotoxin.

In order to identify the mechanism of these apparently cholinomimetic actions, the effects of ranitidine on AChE and BuChE were studied. Ranitidine showed an instantaneous and promptly reversible inhibitory action at concentrations between 0.5 and 30 μM. Double reciprocal plots were prepared and equilibrium dissociation constants calculated. It appears that ranitidine exerts an inhibition of the "mixed" type on both AChE and BuChE, but the dissociation constants for BuChE were markedly higher than those for AChE. Since AChE inhibition occurs in the same concentration range potentiating the twitch responses on the ileal myenteric preparation, it may explain the cholinomimetic effect of ranitidine.

Ranitidine, *N*-[2[[[5-[(dimethylaminomethyl)]-2-furanyl]methyl]thio]ethyl] - *N'* - methyl - 2 - nitro - 1, 1 - ethenediamine (Fig. 1), is a potent histamine H_2-receptor antagonist [1]. It exerts powerful inhibitory effects on gastric acid secretion in animals and man [2–4].

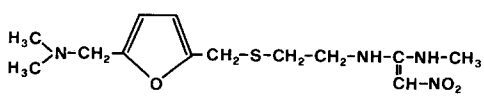

Fig. 1. Chemical structure of ranitidine.

Bertaccini and Coruzzi [5] reported that ranitidine also exerts cholinergic-like effects at the same concentrations which block H_2-receptors. In particular it has been observed that ranitidine induces atropine-sensitive contractions in the lower esophageal sphincter of different animal species and in rat stomach and colon, as well as potentiating the stimulant effect of acetylcholine (ACh)*.

In the present paper, the effects of ranitidine were investigated on the ileal myenteric plexus preparation which is particularly sensitive to muscarinic agonists and antagonists [6] in order to define these apparently cholinomimetic actions. Assuming the possibility of an indirect effect through cholinesterase inhibition, a study of the effect of ranitidine on acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) was also carried out. While our experiments were in progress, Hansen and Bertl [7] reported that ranitidine indeed inhibits human erythrocyte and gastric mucosa AChE as well as human serum BuChE. In our study, however, we also investigated the kinetics of the cholinesterase inhibition of ranitidine and compared its pharmacological effects with those of eserine.

* Abbreviations used: ACh, acetylcholine; AChE, acetylcholinesterase; ATCh, acetylthiocholine; BuChE, butyrylcholinesterase; BuTCh, butyrylthiocholine; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); GABA, γ-aminobutyric acid.

MATERIALS AND METHODS

Ileal myenteric preparation. Guinea-pigs of both sexes weighing 300–500 g, purchased from Morini Farm, were stunned and bled. The small intestine was removed and cut into segments 3–4 cm in length, after which the longitudinal muscle, with the myenteric plexus still attached, was gently separated from the underlying circular muscle [6].

The dissected muscle strip was suspended under tension of 1 g in a 10 ml organ bath containing Krebs–bicarbonate solution (118 mM NaCl; 4.7 mM KCl; 2.5 mM $CaCl_2$; 1.2 mM $MgCl_2$; 1.2 mM NaH_2PO_4; 25 mM $NaHCO_3$ and 11 mM glucose) and bubbled with 95% O_2 and 5% CO_2.

Isometric contractions were elicited by continuous stimulation by supramaximal (10 V/cm) square wave pulses of 1 msec duration at a frequency of 0.1 Hz via two platinum ring electrodes mounted 4 cm apart at the top and bottom of the bath. The contractions were recorded by an isometric force-transducer coupled to a U. Basile 7050 recorder (Comerio, Italy). In a few experiments, stimulation frequencies of 1, 5 and 10 Hz were used. After obtaining constant twitch responses, the drugs were added to the bath fluid and the ensuing changes in the contraction amplitude were measured. The drug was washed out 1–2 min later.

Cholinesterase assay. Purified AChE (1000 units/mg lyophilized material) from *Electrophorus electricus* was purchased from Boehringer Mannheim

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Gmbh and purified horse serum BuChE (13.3 units/mg protein) from Sigma Chemical Co., St Louis, MO, U.S.A.. Human erythrocyte AChE was obtained from 0.1 ml heparinized blood. The erythrocytes were washed 3 times by centrifugation with 9 ml of saline solution and resuspended in 20 ml of 50 mM sodium phosphate buffer, pH 7.2 in saline.

Cholinesterase activity was measured at 25° and pH 7.2 by the photometric method of Ellman *et al.* [8] using acetylthiocholine (ATCh) or butyrylthiocholine (BuTCh) as substrates. In the standard procedure, to 50 μl aliquots of electric eel AChE or horse serum BuChE, equal to 0.062 and 0.050 units of enzymic activity respectively, in 50 mM sodium phosphate buffer, pH 7.2, 2.75 ml of a 0.25 mM of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in buffer were added. In the case of human erythrocyte AChE, 0.9 ml of the washed red blood cell suspension, containing 0.01–0.02 units of enzymic activity, were used with 1.9 ml of DTNB solution. 0.1 ml of inhibitor in buffer or buffer alone were then added to the enzyme. The samples were preincubated at 25° for the appropriate times prior to the addition of 100 μl of ATCh, or BuTCh, to start the hydrolysis. ATCh and BuTCh were purchased from Boehringer, Mannheim, Gmbh. The variations in optical absorbance at 412 nm were measured at 30 or 60 sec intervals by means of a Beckman, Acta III, spectrophotometer equipped with a chart recorder. All concentration values reported in the paper refer to final concentrations present in the 3 ml volume sample used in the activity assay.

In order to evaluate the influence of the contact time between enzyme and inhibitor on cholinesterase inhibition by ranitidine, aliquots of eel AChE were preincubated with a fixed concentration of the drug for varying times before the enzymic hydrolysis was started by adding the substrate (0.5 mM ATCh). The final concentration of ranitidine was 3 μM. The change in optical absorbance was measured within 30 sec from the beginning of the reaction.

The reversibility of the inhibitory action of ranitidine was evaluated according to the following procedure: two 50 μl portions of a solution containing electric eel AChE (1 unit/ml), preincubated in the presence of 30 μm ranitidine, were diluted to 2.9 ml, the one with DTNB in 30 μM ranitidine and the other with DTNB in buffer. Both samples were then assayed for enzymic activity after the addition of 0.5 mM ATCh, at various times after dilution.

Drugs used. GABA, atropine sulphate, morphine hydrochloride, eserine sulphate (Sigma), acetylcholine chloride and norepinephrine bitartrate (Merck), nicotine sulphate (BDH), tetrodotoxin (Sankyo). Ranitidine was a gift from Glaxo S.p.A., Verona, Italy.

RESULTS

Ileal myenteric plexus preparation. Ranitidine added to the perfusion fluid at concentrations from 1 to 100 μM brought about a dose-dependent increase in the electrically induced contractions of longitudinal muscle strip, as shown in Fig. 2. The twitch response elicited by other stimulation frequencies (1, 5, 10 Hz) were similarly affected by the drug. At higher concentrations (0.3–3 mM) ranitidine also caused irregular slow contractions of the unstimulated ileal preparation.

As illustrated by the inset of Fig. 2, the increase in the twitch response caused by ranitidine was immediate, reached a peak after a few seconds and was maintained until the drug was removed. No tachiphylaxis was observed.

Eserine at a concentration of 0.03 μM also potentiated the twitch response but with a different time-course. As shown in Fig. 3 the onset of eserine potentiation was slow, the peak effect was attained after several minutes and the effect was still evident after several washings.

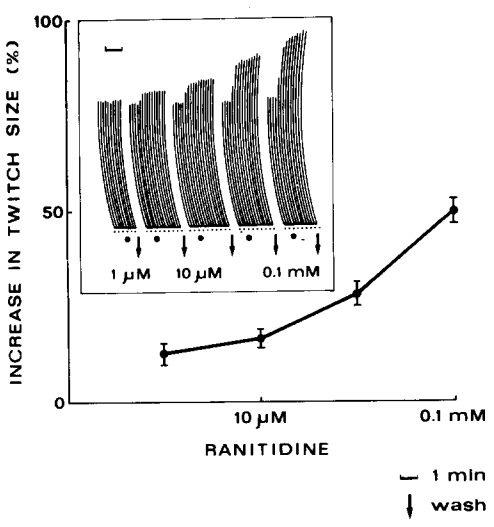

Fig. 2. Dose–effect relationship of ranitidine potentiating action on electrically-evoked contractions of guinea-pig ileum myenteric plexus longitudinal muscle strip. (. . . . .) = field stimulation: 1 msec, 0.1 Hz, supramaximal voltage (70 V). *Inset:* typical experiment showing the effect of graded doses of ranitidine on the same experimental conditions.

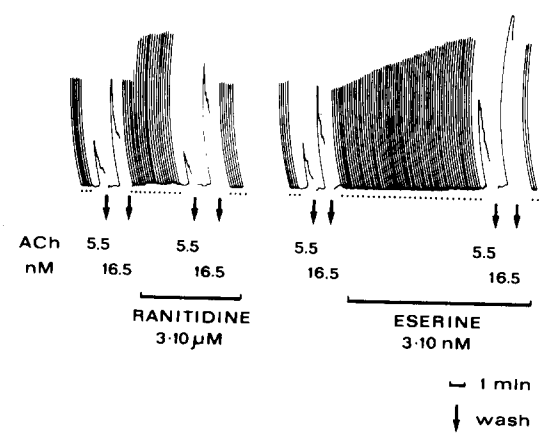

Fig. 3. Electrically stimulated longitudinal muscle strip (1 msec, 0.1 Hz, 70 V). Potentiating effect of ranitidine and eserine on the twitch length. In contrast to eserine, ranitidine is ineffective in enhancing the contractions caused by the administration of ACh.

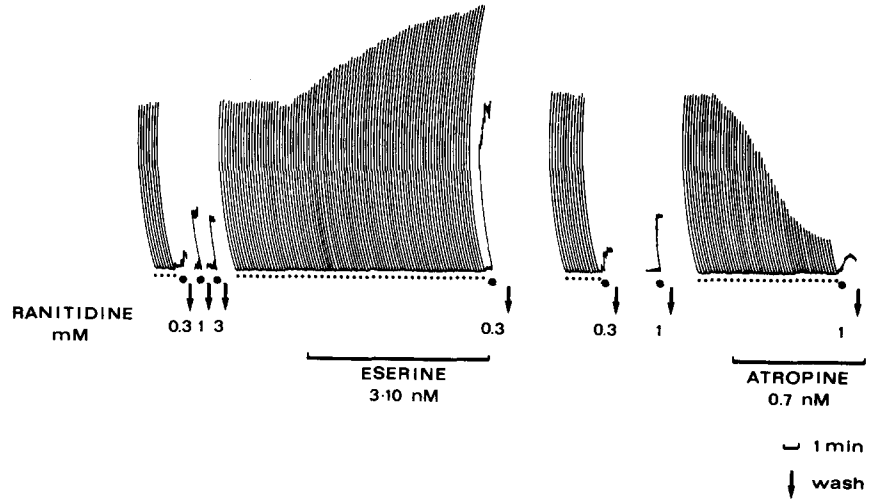

Fig. 4. Electrically stimulated longitudinal muscle strip (1 msec, 0.1 Hz, 70 V). The effect of ranitidine on the unstimulated organ is strongly enhanced by eserine and antagonized by atropine.

The contractions induced by large concentrations of ranitidine on the unstimulated preparation (Fig. 4) were potentiated by eserine sulphate (0.1 μM) and blocked by atropine sulphate (0.7 nM). Hexamethonium at a concentration (10 μM) able to prevent the stimulating action of nicotine (1.5 μM) was ineffective. On the other hand, the contractions were prevented by perfusion with tetrodotoxin (3 μM).

The facilitatory effect of ranitidine on the twitch response was neither affected by desensitization of 5-HT receptors accomplished by perfusing the ileal muscle preparation with 1 μM 5-HT for 10 min, nor by the presence of the receptor blocker yohimbine at a concentration of 10 μM. However, yohimbine by itself slightly potentiated the twitch response. The facilitatory effect of ranitidine on the twitch response also occurred when the contractions of the ileal preparation were depressed by adding either morphine (100 μM), norepinephrine (10 μm) or GABA (100 μM) to the bath, or on the other hand by increasing Mg^{2+} concentration in the Krebs solution to 4.5 mM.

Figure 3 also shows that the administration of ACh to the unstimulated longitudinal muscle preparation induced dose-dependent contractions. These contractions were not potentiated by the presence of ranitidine at a concentration enhancing the electrically-evoked twitch responses. Conversely an equipotent concentration of eserine strongly potentiated both the twitch response and the contractions induced by ACh.

Cholinesterase inhibition. The anticholinesterase activity of ranitidine was directly evaluated in preparations of electric eel and human erythrocyte AChE and horse serum BuChE.

Our results showed that ranitidine action on cholinesterases is time-independent. This was ascertained by preincubating aliquots of eel AChE with a fixed concentration of ranitidine for periods of time varying from 0 to 40 min before adding ATCh (0.5 mM) to measure enzymic activity. It was seen that the initial AChE inhibition (57 ±5% caused by 3 μM ranitidine) remained practically unmodified in all samples tested, independent of preincubation duration.

In separate experiments the progression of electric eel inhibition by 2 μM ranitidine was evaluated in the presence of substrate (0.5 mM ATCh) by continuously recording the change in optical absorbance for 30 min after the beginning of the reaction. The spectrophotometric recording thus obtained* demonstrated that the increase in optical absorbance was linear for the period of time investigated, which is to say that the inhibitory action of the drug remained constant during ATCh hydrolysis. Under the same experimental conditions, inhibition by 0.5 μM eserine, unlike ranitidine, caused changes in optical absorbance resulting in a curved line with a downward concavity*. In this case, therefore, a progressive increase in AChE inhibition occurred, continuing also during ATCh hydrolysis.

Table 1 illustrates the prompt reversibility of ranitidine inhibition after a 60-fold dilution of the incubation mixture as described in the section Materials

Table 1. Reversibility of electric eel AChE inhibition by ranitidine following dilution of the drug

Time after dilution (min)	AChE inhibition (%) 30 μM ranitidine	Dilution with buffer*
0.5	89 ± 5	8 ± 2
1.5	90 ± 5	6 ± 4
5.5	87 ± 4	7 ± 4
10.5	87 ± 3	6 ± 3
20.5	88 ± 3	7 ± 2
30.5	89 ± 4	6 ± 2
40.5	87 ± 4	8 ± 4

The experimental procedure was described under Methods. The activity assay was carried out at 25° in 50 mM sodium phosphate buffer, pH 7.2, in the presence of 0.5 mM ATCh. The values represent the means ± S.E.M. of 3 separate determinations in duplicate.

* Final ranitidine concentration = 0.5 μM.

* Data not shown.

and Methods. It can be seen that AChE inhibition by ranitidine drops from 89 to 8% in the first assay performed within 30 sec after dilution.

The reversibility of AChE inhibition by ranitidine was also assayed in the presence of substrate by the following procedure. An aliquot of electric eel AChE was incubated at 25° for 15 min in the presence of 30 μM ranitidine and 0.5 mM ATCh. At the end of the incubation, 50 μl portions of the inhibited enzyme were rapidly diluted 60 times in the spectrophotometer cuvette with DTNB in 0.5 mM ATCh. The changes in optical absorbance were continuously recorded for 30 min. No variation in the reaction velocity during this period was found, which demonstrates that the enzyme had recovered its maximal theoretical activity immediately after dilution.

Clearly, the association and dissociation rates of AChE and ranitidine are so rapid that they cannot be detected by our method.

In order to determine the type of cholinesterase inhibition by ranitidine and the inhibition constants, saturation experiments with increasing substrate concentrations were carried out. The double reciprocal plots [9] obtained from the experimental data were linear in all cases examined, as shown in Fig. 5, but they clearly presented different patterns depending on the enzyme tested. The inhibition of electric eel and human erythrocyte AChE gave similar results and only those obtained with electric eel AChE are shown in Fig. 5a. AChE inhibition in the presence of different concentrations of ranitidine produced straight lines characterized by increasing slopes and increasing intercepts on the $1/V$ axis. The slope variations however were very small and the lines derived from the reciprocal plots appeared, particularly at low inhibitor concentrations, almost parallel to the line of the uninhibited enzyme. Both K_m and V_{max} were therefore affected by the drug. These results are consistent with an inhibition of the "mixed" type, which presents characteristics common to both "competitive" and "non-competitive" inhibition [10].

According to current ideas on enzymic inhibition [10, 11], "mixed" inhibition can occur when a reversible inhibitor combines with a free enzyme (E) and the enzyme–substrate complex (ES) with different affinities ($K_i \neq K_i'$). In the case of cholinesterases, however, the catalytic mechanism requires the formation from the reversible ES complex of a short-lived acetyl derivative of the enzyme [12]. A "mixed" inhibitor of cholinesterase, therefore, could develop its inhibitory action by interacting with either one or both enzyme combinations.

The equilibrium dissociation constant for ranitidine-free enzyme combination, K_i, and that for the interaction of the drug with ES or acetyl enzyme, K_i', were calculated graphically from the reciprocal plots (see legend in Table 2) according to the equation [10].

$$\frac{1}{V} = \frac{K_m}{V_{max}[S]}\left(1 + \frac{[i]}{K_i}\right) + \frac{1}{V_{max}}\left(1 + \frac{[i]}{K_i'}\right)$$

and are shown in Table 2.

It can be seen that K_i' values for electric eel and erythrocyte AChE were markedly lower than the corresponding K_i values in agreement with the primarily "uncompetitive" character of ranitidine action on AChE.

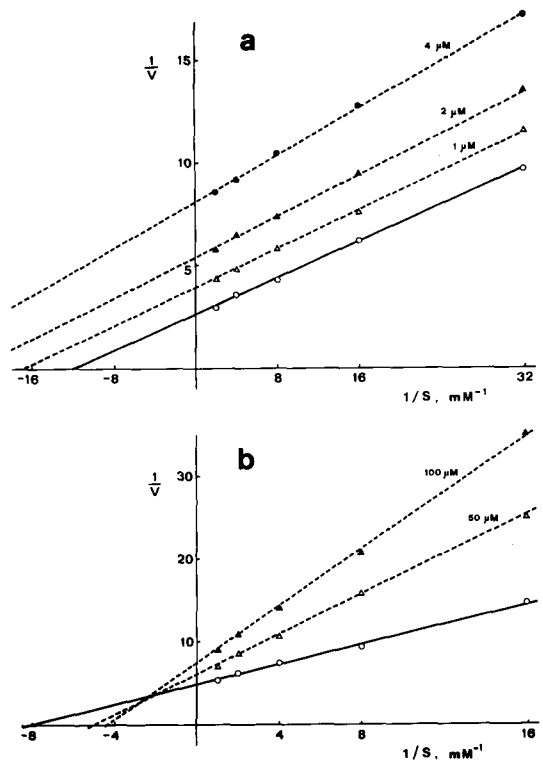

Fig. 5. Inhibition of electric eel AChE (a) and horse serum BuChE (b) by ranitidine: double reciprocal plots. The activity assays were carried out according to the procedure described in Materials and Methods. Aliquots of 50 μl of the enzymes were preincubated with the inhibitor or with buffer alone at 25° for 1 min prior to adding: (a) ATCh in the range of concentrations S: 0.031–0.5 mM; (b) BuTCh in the range of concentrations S: 0.031–1 mM. $V = \Delta$ absorbance/min. (a): buffer (○——○), 1 μM (△- - -△), 2 μM (▲- - -▲), 4 μM (●- - -●) ranitidine hydrochloride; (b): buffer (○——○), 50 μM (△- - -△), 100 μM (▲- - -▲) ranitidine hydrochloride. The points on the graph represent the mean values of 3 separate experiments performed in duplicate.

The inhibition of horse serum BuChE also presented "mixed" characteristics. Figure 5b shows that the lines of the inhibited and uninhibited enzyme in the reciprocal plot intersect above the $1/S$ axis, revealing an increase in the K_m of the enzyme as a result of ranitidine action. The K_i and K_i' of BuChE, calculated according to the same equation, are shown in Table 2. BuChE inhibition constants are much higher than the corresponding values for AChE. Furthermore, K_i is lower than K_i' which would indicate the prevalence of a "competitive" action of ranitidine on this enzyme.

DISCUSSION

Our experiments indicate that the stimulatory effect of ranitidine on the ileal myenteric plexus preparation can be considered cholinergic in nature. It is known [6] that the twitch-response elicited in

Table 2. K_i and K_i' constants of ranitidine for cholinesterases from different sources

Dissociation constants	Electric eel (μM)*	Human erythrocytes (μM)*	Horse serum (μM)†
K_i	12.8 ± 3.2	9.1 ± 2.0	53 ± 8.2
K_i'	1.9 ± 0.4	1.1 ± 0.2	177.0 ± 32.0

The equilibrium dissociation constants K_i and K_i' were calculated from double reciprocal plots (see Fig. 5), by replotting the intercepts on $1/V$ axis and the slopes against 3–4 different ranitidine concentrations: the intercepts on base line gave K_i' and K_i respectively [10]. The conditions of the activity assay were those reported in the legend of Fig. 5.

* ATCh (0.031–0.5 mM) as substrate.

† BuTCh (0.031–1 mM) as substrate.

the longitudinal muscle strip by electrical stimulation is mediated by ACh released from the myenteric plexus. An enhancement of the twitch-response can therefore be achieved by increasing ACh release or by potentiating its postsynaptic action. The demonstration that ranitidine inhibits cholinesterases at the same concentrations at which it is active on the ileal myenteric preparation favors the second possibility, in which case ranitidine would appear to act according to a mechanism similar to that of eserine. The different time-courses of the reactions between the two cholinesterase inhibitors and the enzyme (see below) can explain the differences in onset and duration of eserine and ranitidine action on the twitch-response, and the lack of potentiation by ranitidine of the contractions induced by exogenous ACh.

Further evidence of an indirect cholinomimetic action on the part of ranitidine derives from the experiments on the unstimulated ileal preparation. The blockade of ranitidine-induced contractions with atropine and the potentiation with eserine confirm their cholinergic nature, while the blockade with tetrodotoxin demonstrates that they depend upon nervous activity [13].

Ranitidine inhibitory action on cholinesterases develops very rapidly and is promptly reversible on dilution. By contrast, it has been shown [14, 15] that the inhibitory action of eserine is progressive. Likewise, the inhibitory action of eserine on AChE is not as readily reversible as that of ranitidine. When AChE is preincubated with eserine, it takes the enzyme approximately 38 min to regain half maximum activity after large dilution [16].

Ranitidine anticholinesterase activity appears to be rather selectively directed towards AChE as demonstrated by the finding that dissociation constants of ranitidine-BuChE are markedly higher than the corresponding constants of ranitidine-AChE, which agrees with a previous observation [7].

The effect of the substrate on the inhibitory action of ranitidine is complex and there are clear differences with regard to AChE and BuChE. The inhibition of electric eel and erythrocyte AChE is slightly enhanced by increasing substrate concentrations while, on the other hand, the inhibition of horse serum BuChE is clearly decreased in the same conditions.

The rather strong action of ranitidine on AChE appears principally to be due to a non-competitive high affinity interaction of the drug with one or both of the enzyme–substrate intermediates formed during the catalytic process, rather than to a competition with the substrate at the free enzyme. In this respect our results differ from those reported by Hansen and Bertl, who define AChE inhibition by ranitidine as competitive [7]. From our data it is not possible to establish whether ranitidine binds to the reversible enzyme–substrate complex or to the acetyl enzyme or to both of them. However, the most likely hypothesis is that the drug interacts with the latter intermediate, blocking its deacetylation. Such a mechanism has already been demonstrated for some aminoalcohols and derivatives which inhibit AChE in a mixed, competitive and non-competitive manner [17–19]. On the other hand, no anticholinesterase agent has been shown, to our knowledge, to block the breakdown of the AChE–substrate complex.

Ranitidine interacts very feebly with BuChE, showing in this case higher affinity for the free enzyme than for the subsequent intermediates. This confers on the inhibition a prevalence of "competitive" character.

In conclusion, the fact that ranitidine inhibits AChE may explain its effect on the ileal myenteric preparation and the reported cholinomimetic effects in animal and man [2]. However, the prompt reversibility of the inhibition may in turn explain the lack of disturbing cholinergic side effects in patients treated with ranitidine.

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