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Inhibition of human plasma cholinesterase and erythrocyte acetylcholinesterase by nondepolarizing neuromuscular blocking agents

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Abstract

Purpose. The kinetics of the inhibition of human plasma cholinesterase (ChE) and erythrocyte acetylcholinesterase (AChE) by alcuronium, atracurium, *d*-tubocurarine, pancuronium, pipecuronium, and vecuronium were studied in blood drawn from 35 surgical patients.

Methods. The activities of plasma ChE and erythrocyte AChE were determined by the calorimetric method of Ellman et al., using acetylthiocholine as the substrate. Lineweaver-Burk plots and Dixon plots were used for the analysis of the kinetics of both enzymes.

Results. The dissociation constants (K_m) of plasma ChE and erythrocyte AChE were 5.00×10^{-5}M and 5.28×10^{-5}M, respectively, indicating that both enzymes have similar affinity to acetylthiocholine. Both Lineweaver-Burk plots and Dixon plots indicated that the six nondepolarizing neuromuscular blocking agents (NMBAs) at different concentrations induce linear mixed-type inhibition. The apparent inhibition constants (K_i) of pancuronium (8.72×10^{-8}M) and vecuronium (3.53×10^{-7}M) for plasma ChE inhibition were lower than that of neostigmine (7.36×10^{-7}M), whereas those of the six nondepolarizing NMBAs for erythrocyte AChE were markedly higher than that of neostigmine.

Conclusions. Both plasma ChE and erythrocyte AChE were inhibited by six nondepolarizing NMBAs, and the pattern of inhibition of both enzymes was of mixed type. The inhibitory potencies of pancuronium and vecuronium for plasma ChE were larger than that of neostigmine, whereas those of the six nondepolarizing NMBAs for erythrocyte AChE were markedly lower than that of neostigmine. The rank order of relative potency for plasma ChE was pancuronium > vecuronium > pipecuronium > alcuronium > *d*-tubocurarine > atracurium.

Key words: Plasma cholinesterase, Erythrocyte acetylcholinesterase, Nondepolarizing neuromuscular blocking agents, Lineweaver-Burk plot, Kinetics of cholinesterase inhibition

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Introduction

Nondepolarizing neuromuscular (NM) blocking agents (NMBAs) inhibit human plasma cholinesterase (ChE; EC 3.1.1.8) and acetylcholinesterase (AChE; EC 3.1.1.7) present at the NM junction [1]. Inhibition of AChE activity by nondepolarizing NMBAs at the NM junction will increase the amount of ACh available there. This will result in the activation of the ACh receptor, and therefore nondepolarizing NMBAs will partially antagonize their own NM activity. On the other hand, a decrease in plasma ChE activities will impair the metabolism of drugs that are inactivated by this enzyme, such as succinylcholine and procaine.

Kinetic studies using natural and synthetic substrates have demonstrated that there are several different binding sites for ACh or inhibitors of AChE, including the active catalytic site and peripheral allosteric site, and the binding of certain drugs with the catalytic site is capable of modulating the activity of the site through conformational changes in AChE [2–4]. A nondepolarizing NMBA, gallamine, is bound exclusively to the active ACh binding site, exhibiting pure competitive inhibition [2,5,6]. On the other hand, *d*-tubocurarine is not purely competitive with ACh in binding at the active site, but is bound to a peripheral site. The inhibition pattern of *d*-tubocurarine is of mixed type (partial competitive and noncompetitive inhibition) [5,7]. However, the nature of the catalytic interactions of clinically available nondepolarizing NMBAs, including benzylisoquinolinium and the steroidal compounds with human plasma ChE and AChE activities, has not been reported.

This study was undertaken to investigate the possible inhibitory effects of alcuronium, atracurium, pancuronium, pipecuronium, vecuronium or *d*-tubocurarine, and to analyze the kinetics of their interactions with human plasma ChE and erythrocyte AChE.

Materials and methods

After approval had been granted by the ethics committee of our institution, informed consent was obtained from 35 surgical patients of both sexes, aged 22 to 63 years, classified as American Society of Anesthesiologists physical status 1 or 2. Patients with a history of NM, renal, or hepatic disease and those taking drugs known to interfere with NM function were excluded.

Premedication consisted of meperidine 35–70 mg and atropine 0.5 mg, given intramuscularly 60 min before the induction of anesthesia. Before the induction of anesthesia, 10 ml of venous blood was obtained in heparin and stored at 0°C until the assay. Plasma ChE and erythrocyte AChE activities were determined by the calorimetric method of Ellman et al. [8], using 5×10^{-3} M acetylthiocholine (AtCh) as the substrate. All the reagents were obtained from Boehringer Mannheim. (Mannheim, Germany) as a test combination kit (cholinesterase color "BMY").

To determine plasma ChE activities, 2 to 3 ml of blood samples placed in a polyethylene tube were centrifuged at 3000 rpm for 10 min after measurement of their hematocrit, and the plasma was collected. After 20 µl of plasma was mixed with 3.0 ml of 5.2×10^{-2} M phosphate buffer (pH 7.2) plus 2.6×10^{-4} M 5,5′-dithiobis-2-nitrobenzonic acid (DTNB), the reaction was started by adding 100 µl of 5×10^{-3} M AtCh.

Erythrocytes were separated by centrifugation from another 200-µl blood sample at 3000 rpm for 10 min at 4°C, and then hemolysed with 1.0 ml of distilled water. After 20 µl of hemolysate was added to 3 ml of a solution consisting of 2.98 ml of 5.2×10^{-2} M phosphate buffer (pH 7.2) and 2.62×10^{-4} M DTNB plus 20 µl of 0.37% quinidine sulfate solution, a specific plasma ChE inhibitor, the reaction was started by adding 100 µl of 5×10^{-3} M AtCh. The rate of change in optical density (OD) at 410 nm was measured four times every 30 s at 25°C with a spectrophotometer (Uvidec 77, Japan Spectroscopic, Tokyo, Japan).

Four or five different concentrations of the NMBAs were incubated with plasma ChE or erythrocyte AChE in buffer for 5 min at 25°C before the addition of the substrate. All determinations were performed in duplicate, and the mean values were used for subsequent calculations. The enzyme activities were calculated as follows:

$$\text{Plasma ChE activities (IU·l}^{-1}) = [2 \times (\Delta E - \Delta E') / \varepsilon] \times (V_t / V_l) \times 10^6 = (\Delta E - \Delta E') \times 23\,009$$

$$\text{Erythrocyte AChE activities (IU·l}^{-1}) = [2 \times (\Delta E - \Delta E') / \varepsilon] \times (V_t / V_l) \times 10^6 \times D = (\Delta E - \Delta E') \times 23\,009 \times (1000 + 2 \times \text{Ht}) / (2 \times \text{Ht})$$

where ΔE = mean change in absorbance at 410 nm for 30 s; $\Delta E'$ = mean change in absorbance at 410 nm for 30 s when 20 µl of distilled water is added to the reagents instead of plasma or hemolysates, which indicates the nonenzymatic hydrolysis of the substrate; V_t = volume of reaction mixture (3120 µl); V_p = volume of plasma (20 µl); V_l = volume of hemolysates (20 µl); Ht = hematocrit of blood sample (%); D = dilution rate of erythrocytes, which is $(1000 + 2 \times \text{Ht}) / 2 \times \text{Ht}$, and $\varepsilon = 1.356 \times 10^4 \text{l·mol}^{-1}\text{·cm}^{-1}$ (molar absorptivity at 410 nm).

The sources of the nondepolarizing NMBAs and neostigmine used in this study were as follows: alcuronium chloride, F. Hoffman-La Roche, Basel, Switzerland; atracurium besylate (Glaxo Wellcome, Research Triangle Park, NC, USA; *d*-tubocurarine chloride, Wako Pure Chemical, Osaka, Japan; pancuronium bromide, Organon Teknika, Turnhout, Belgium; pipecuronium bromide, Organon Teknika; vecuronium bromide, Organon Teknika; and neostigmine bromide, Wako Pure Chemical. These reagents were dissolved in distilled water.

Lineweaver-Burk plots [9] and Dixon plots [10] were used for analysis of the kinetics of both enzymes.

The figures were taken from statistical calculation of four or five points in each series using a linear least-squares approximation. The results shown in the figures are expressed as means ±SE. The values in the text and table are expressed as medians.

Results

Lineweaver-Burk and Dixon plots for the inhibition of erythrocyte AChE activity by vecuronium for the hydrolysis of AtCh are shown in Fig. 1A and 2A, respectively. The intersection point of each straight line lies in the third quadrant, indicating that the inhibition was of the linear mixed type. The mixed type inhibition of the erythrocyte AChE was confirmed by the secondary replot of the slope from the Dixon plot versus 1 / AtCh (Fig. 2B). The inhibition pattern observed for six nondepolarizing NMBAs tested with both enzymes in the same manner was of mixed type.

The dissociation constants (K_m) for plasma ChE and erythrocyte AChE from the regression equations over the range of substrate concentrations from 6×10^{-5} M to 2×10^{-4} M at zero concentration of nondepolarizing NMBA were 5.00×10^{-5} M and 5.28×10^{-5} M, respectively.

The apparent inhibition constant (K_i) value of erythrocyte AChE for vecuronium was estimated as 2.30×10^{-6} M from the secondary replot of the slope versus vecuronium concentration from a Lineweaver-Burk plot for the determination of K_i (Fig. 1B). The K_i values in both enzymes for the six nondepolarizing NMBAs were obtained in a similar manner, and the results are

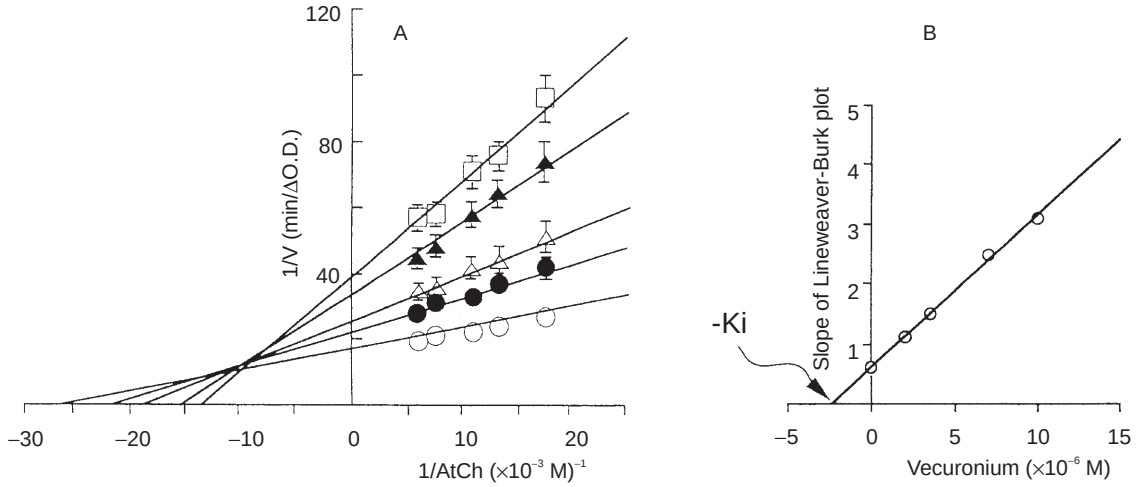

Fig. 1. Typical pattern of erythrocyte AChE inhibition by nondepolarizing neuromuscular blocking agent produced by vecuronium. **A** Lineweaver-Burk plots for the inhibition by vecuronium of the hydrolysis of acetylthiocholine (AtCh) by erythrocyte AChE. The ordinate is the reciprocal of the velocity of AtCh hydrolysis, expressed as min/ΔOD (optical density). The abscissa is the reciprocal of the concentration of AtCh (6×10^{-5}M to 2×10^{-4}M). *Open circles*, no vecuronium; *filled circles*, 2×10^{-6}M; *open triangles*, 3×10^{-6}M; *filled triangles*, 7×10^{-6}M; *open squares*, 1×10^{-5}M vecuronium. Each point is the mean ±SE of 5 independent determinations. **B** Secondary replot of the slope from Lineweaver-Burk plot vs. vecuronium concentration for the determination of K_i

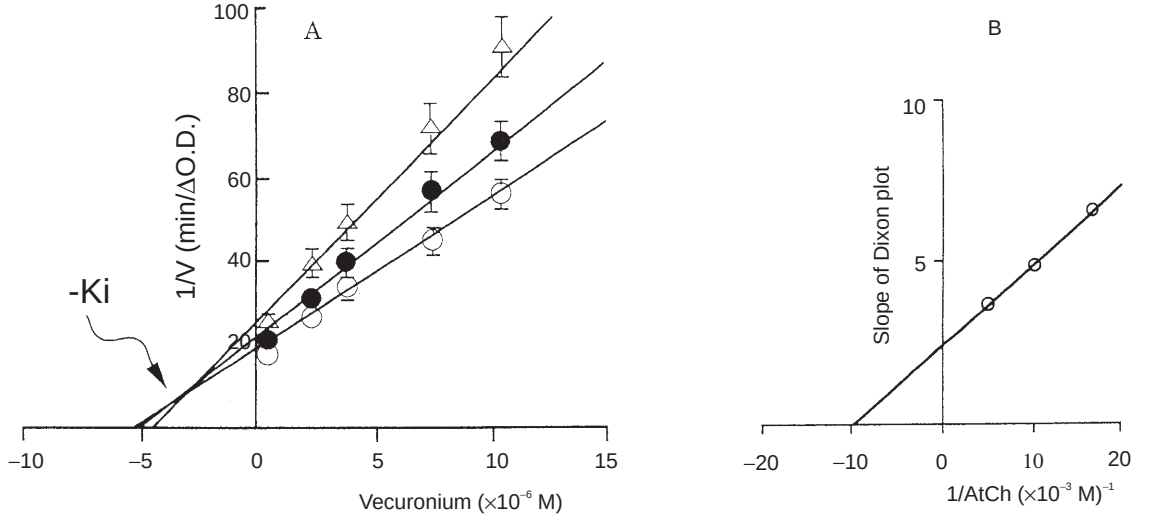

Fig. 2. Typical pattern of erythrocyte AChE inhibition by nondepolarizing neuromuscular blocking agent produced by vecuronium. **A** Dixon plots for the inhibition of erythrocyte AChE by vecuronium. The ordinate is the reciprocal of the velocity of acetylthiocholine (AtCh) hydrolysis, expressed as min/ΔOD (optical density). The abscissa is vecuronium concentration. Three different fixed concentrations of AtCh: *open circles*, 2×10^{-4}M; *filled circles*, 1×10^{-4}M; *open triangles*, 6×10^{-5}M in the presence of 2×10^{-6}M to 1×10^{-5}M vecuronium concentration. Each point represents the mean ±SE of 5 independent determinations. **B** Replotting of the slope of Dixon plot vs. 1 / AtCh

summarized in Table 1. The inhibitory potencies of the six nondepolarizing NMBAs equipotent to neostigmine (= 1000) are also shown in Table 1. The potencies of pancuronium and vecuronium for plasma ChE were higher than that of neostigmine, whereas those of the six nondepolarizing NMBAs for erythrocyte AChE were markedly lower than that of neostigmine. The rank order of relative potency for plasma ChE was pancuronium > vecuronium > pipecuronium > alcuronium > *d*-tubocurarine > atracurium.

Table 1. Inhibition constants (K_i) for plasma cholinesterase (ChE) and erythrocyte acetylcholinesterase (AChE), and relative potency for inhibition by six nondepolarizing neuromuscular blocking agents equipotent to neostigmine (= 1000)

Agent	K_i (M) Plasma ChE	Relative potency	K_i (M) Erythrocyte AChE	Relative potency
Pancuronium	8.72×10^{-8} ($n = 5$)	8400	7.41×10^{-6} ($n = 5$)	9
Vecuronium	3.53×10^{-7} ($n = 5$)	2085	2.30×10^{-6} ($n = 5$)	27
Pipecuronium	3.34×10^{-6} ($n = 5$)	220	5.88×10^{-6} ($n = 5$)	11
Alcuronium	9.47×10^{-6} ($n = 5$)	78	3.25×10^{-5} ($n = 5$)	2
d-Tubocurarine	2.66×10^{-5} ($n = 5$)	28	8.19×10^{-5} ($n = 5$)	1
Atracurium	3.08×10^{-5} ($n = 5$)	24	7.81×10^{-6} ($n = 5$)	8
Neostigmine	7.36×10^{-7} ($n = 5$)	1000	6.31×10^{-8} ($n = 5$)	1000

Discussion

According to Lineweaver-Burk analysis, inhibition of plasma ChE and erythrocyte AChE by six nondepolarizing NMBAs decreased progressively with increase in the AtCh concentration, indicating that the effects of all six were reversible [9]. Moreover, these results suggest that the six nondepolarizing NMBAs, at different concentrations, induced linear mixed-type inhibitions that are partially competitive and partially noncompetitive. The partially competitive type of inhibition of the enzymes was confirmed by the secondary replot of the slope from the Dixon plot against the reciprocal of AtCh (Fig. 2B), since the straight line did not go through the origin of the replot as it would in the case of purely competitive inhibition. The noncompetitive nature of the inhibition was suggested by secondary slope replot of the Lineweaver-Burk plot against concentrations of the nondepolarizing NMBA (Fig. 1B), in which the plots showed straight lines rather than curved lines, as would be the case in partially noncompetitive inhibition [10]. AChE is affected by increasing the concentration of the six nondepolarizing NMBAs, suggesting that these NMBAs bind to the catalytic anionic site (α) and the peripheral allosteric site (β-anionic site) on plasma ChE and erythrocyte AChE [3,11,12].

In the present study, the K_m values of human plasma ChE (5.28×10^{-5}M) and erythrocyte AChE (5.00×10^{-5}M) for AtCh were similar, indicating that both enzymes have almost the same affinity for the substrate. These values are similar to those found by Tsukawaki et al. [13] in their studies of trimethaphan on human plasma ChE (6.6×10^{-5}M) and erythrocyte AChE (7.6×10^{-5}M) for AtCh. On the other hand, Al-Jafari et al. [12] have shown that K_m is 13.2×10^{-5}M for human erythrocyte AChE, using AtCh as the substrate.

Plasma ChE, erythrocyte AChE, and AChE that is present at the NM junction exist as polymers of catalytic subunits [14]. The amino acid sequences obtained from five tryptic peptides of human erythrocyte AChE showed no homology with human plasma ChE. However, when these sequences are compared with those of other mammalian AChEs, approximately 88% sequence identity is observed [15]. Additionally, the amino acid sequence of fetal bovine serum AChE showed overall homologies of 90% with human AChE and 50% with human plasma ChE in these catalytic subunits [16]. The similarities and any differences in their catalytic functions should be reflected in the corresponding similarities and differences in the structural domains of their catalytic subunits [16]. Therefore, the activity produced by substituting erythrocyte AChE for AChE at the NM junction may be suitable.

In the present study, pancuronium ($K_i = 8.72 \times 10^{-8}$M) was a potent inhibitor of plasma ChE activity and was almost eight times more potent than neostigmine ($K_i = 7.36 \times 10^{-7}$M). On the other hand, the inhibitory effect of pancuronium on erythrocyte AChE activity ($K_i = 7.41 \times 10^{-6}$M) was more than 100 times weaker than that on plasma ChE. Pancuronium has been shown to exert a strong inhibitory effect on plasma ChE in vitro, and its inhibitory effect is selective on plasma ChE activity, but the results in vivo have varied [17,18]. Stovner et al. [17] reported that $0.1\,mg \cdot kg^{-1}$ of pancuronium reduced the enzymatic activity of plasma ChE to 60% to 80% after control for 3 min. Stoeling [18] showed that $0.1\,mg \cdot kg^{-1}$ of pancuronium produced no inhibition of plasma ChE activity. The reasons for these differences are not apparent. However, it is clinically evident that when a subparalyzing dose of pancuronium, usually $0.01\,mg \cdot kg^{-1}$, is given before succinylcholine to prevent fasciculations and muscle pain, it may cause more than 50% inhibition of plasma ChE activity [1] and prolong the duration of succinylcholine block [1,19]. Other nondepolarizing NMBAs—alcuronium, gallamine, *d*-tubocurarine, and vecuronium—antagonize the block produced by succinylcholine given afterwards [19]. Vecuronium was a relatively potent inhibitor of plasma ChE activity ($K_i = 3.53 \times 10^{-7}$M) but was less potent than pancuronium in

the present study. Although vecuronium has a distinct inhibitory effect on plasma ChE activity in vitro, none of its effects in vivo can be attributed to enzyme inhibition.

Mivacurium, a benzylisoquinolinium diester, is rapidly hydrolyzed by plasma ChE at the rate of 70% to 88% of succinylcholine [20,21]. This hydrolysis leads to the short duration of action of mivacurium. Even a very small dose of mivacurium ($10\,\mu g \cdot kg^{-1}$), which is almost 1×10^{-1} of ED_{95}, given during recovery from pancuronium at the point of EMG response recovery to 25% of the control, caused a prolonged NM block [22]. This could partly explain the fact that pancuronium decreases the rate of mivacurium metabolism and hence prolongs the clinical duration of mivacurium [23,24]. From the results of the present study, it may be assumed that pancuronium increases the potency and duration of action of succinylcholine, mivacurium, and local anesthetics of ester type.

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