Acetylcholine, Cholinesterase Activity, and the Aggregability of Human Platelets[1] (34998)

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(Introduced by Kenneth M. Brinkhous)
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Cholinesterase (ChE) activity has been detected in the platelets of certain nonhuman mammals and in other mammalian cell types (1). In contrast to the numerous studies on ChE activity of human erythrocytes, there has been little interest in this enzyme in relation to human platelets probably due to the inability of several workers (1, 2) to detect its presence in these cells. Several different esterases, however, have been demonstrated previously in human platelets, and recently Kilburn and Firkin reported a study of human platelet tributyrinase (3). The presence of cholinesterase activity in human platelets has been reported previously in brief form only (4).

The purpose of this study was to demonstrate the presence of the ChE activity in human platelets, to determine some of its properties, and to investigate the relation of this enzyme, if any, to platelet aggregation.

Methods. Preparation of thrice-washed human platelets has been described (5). Washed platelets were finally resuspended in 0.05 *M* tris (hydroxymethyl) aminomethane buffer (Tris buffer), pH 8.0. Preparation of "light" and "heavy" platelet plasma-membrane fractions has also been described (6); these two fractions, of different density and believed to be of plasma-membrane origin, were isolated by discontinuous sucrose-gradient ultracentrifugation. In the present study, the membrane fractions were dialyzed overnight against Tris buffer, pH 8.0, prior to assay for ChE activity.

ChE activity was assayed by the colorimetric method of Ellman *et al.* (7), with the substitution of 0.05 *M* Tris buffer, pH 8.0, for phosphate buffer. The reaction mixture contained 2.7 ml of platelets at 230,000/mm^3 or of plasma-membrane preparation, 0.3 ml of Tris buffer or test agent, 0.025 ml of 5,-5′-dithiobis (2-nitrobenzoic acid) (DTNB) reagent, and 0.025 ml of 0.075 *M* acetylthiocholine iodide. Test agents, including ChE inhibitors and platelet aggregating agents, were incubated with platelets for a total of 6 min during which time the reaction was followed colorimetrically at 1-min intervals. Data are given in μMU of cholinesterase/2 $\times$ 10^8 platelets or per 100 μg of plasma-membrane protein (1 μmolar unit or μMU of enzyme will hydrolyze 1 μmole of acetylcholine per min at pH 8.0 at 37° as defined by Sigma Chemical Company). Forty determinations of ChE activity were performed on intact platelets from eight donors to obtain baseline values. ChE activity of intact platelets was demonstrated with four different substrates by the potentiometric method of Witter *et al.* (8), but this method was less convenient than that of Ellman *et al.* (7). ChE activity of thrice-washed erythrocytes was studied with erythrocyte concentration adjusted to give ChE activity comparable to that of platelets at a final concentration of 200,000/mm^3.

The macroscopic platelet-aggregation test of Brinkhous *et al.* (9) was used. Aggregation is graded on a scale of 1+ (5 platelets per aggregate) to 4+ (over 100 platelets per aggregate).

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For all test agents,[3] distilled water was used as solvent except for ADP, thrombin, epinephrine, and DTNB for which Tris buffer, pH 7.0, was the solvent and for FDNB and DFDNB which were dissolved in 40% ethanol. Separate control tests for the effect of 40% ethanol on platelets were performed. Thrombin concentration is expressed in Iowa units (U). Collagen suspension, hereafter referred to as collagen, was prepared by the method of Hovig (10) in 0.154 *M* saline.

Results. ChE activity of intact platelets and isolated plasma membranes. The mean values and standard deviations for ChE activity of intact platelets and "light" and "heavy" membrane fractions were determined. For intact platelets the mean value was $8.21 \pm 0.94 \times 10^{-4}$ μMU/2 $\times 10^8$ platelets while for "light" and "heavy" membrane fractions the values were $7.71 \pm 1.45 \times 10^{-4}$ μMU and $7.81 \pm 1.74 \times 10^{-4}$ μMU/100 μg of membrane protein, respectively.

ChE activity of intact platelets increased linearly with increasing concentration of substrate in the range 10^{-4}–10^{-2} *M*, and with increasing concentration of platelets in the range of 100,000–400,000 platelets/mm^3. Optimal pH for ChE activity was 8.0 but there was relatively little change in ChE activity in the range of pH from 7.1–8.5. ChE activity diminished sharply at pH values greater than 8.5. The ratio of the amount of test substrate utilized to the amount of the standard acetylthiocholine substrate utilized was found to be 0.91 for acetylcholine perchlorate, 1.18 for acetyl-β-methylcholine, and 0.82 for benzylcholine.

[3] Acetylthiocholine iodide, acetylcholine perchlorate, acetyl-β-methylcholine, eserine (physostigmine), crystalline bovine erythrocyte ChE, *d*-tubocurarine, adenosine diphosphate (ADP), L-epinephrine, collagen, 1,5-difluoro-2,4-dinitrobenzene (DFDNB), and 1-fluoro-2,4-dinitrobenzene (FDNB), Sigma Chemical Co., St. Louis, Mo.; imipramine hydrochloride, Geigy Pharmaceuticals, Ardsley, N. Y.; dibucaine, Ruger Chemical Company, Irvington-on-Hudson, N. Y.; procaine hydrochloride, Abbott Laboratories, Chicago, Ill.; and bovine thrombin, Parke, Davis and Co., Detroit, Mich.

TABLE I. Effect of Four Aggregating Agents on ChE Activity of Intact Washed Platelets in the Absence of Added Calcium.

Aggregating agent	Final concn	% Inhibition (—) or stimulation (+) of ChE activity of intact platelets
Thrombin	0.1 U/ml	(—) 100.0
	0.05 U/ml	(—) 29.1
	0.005 U/ml	NE[a]
Collagen	—	(—) 37.4
ADP	$10^{-3}M$	NE
	$10^{-4}M$	(+) 16.3
	$10^{-5}M$	(+) 19.4
	$10^{-6}M$	(+) 13.2
Epinephrine	$10^{-3}M$	(+) 48.6
	$10^{-4}M$	(+) 15.2

[a] NE = no effect.

Effects of aggregating agents on ChE activity. The effects of thrombin, collagen, ADP, and epinephrine on ChE activity of intact platelets is shown in Table I. Thrombin at 0.1 U/ml inhibited completely ChE activity of intact platelets but at 0.005 U/ml it had no effect. Collagen produced 37% inhibition of ChE activity. ADP at 10^{-3} *M* had no effect but at lower concentrations produced slight stimulation of ChE activity of intact platelets. Epinephrine, like ADP, stimulated ChE activity. Calcium at a final concentration of 0.002 *M* or 0.00054 *M* had little effect on the changes produced in ChE activity by ADP or epinephrine, but enhanced moderately the inhibition of ChE activity produced by thrombin or collagen.

Effects of ChE inhibitors on platelet aggregation. Effects of eserine, imipramine, FDNB, DFDNB, dibucaine, *d*-tubocurarine, quinidine and procaine on the ChE activity and aggregability of platelets are shown in Table II. Eserine, imipramine, FDNB, DFDNB, and the higher concentration of procaine produced complete inhibition of ChE activity and, except for eserine, inhibited completely aggregation induced by thrombin, ADP, or collagen. Eserine, however, caused slight enhancement of aggregation induced by these three agents. Dibucaine and *d*-tubocurarine each produced moderate inhibition of ChE activity; dibu-

TABLE II. Effect of Eight Known ChE Inhibitors on Platelet ChE Activity and on Aggregation Induced by Thrombin, ADP, or Collagen.

Test agent[a]	% Inhibition of platelet ChE activity	Effect on platelet aggregation induced by: Thrombin (0.1 U/ml)	ADP (10^{-3} M)	Collagen
Eserine	100	+[b]	+	+
Imipramine	100	—[c]	—	—
FDNB	100	—	—	—
DFDNB	100	—	—	—
Procaine 1×10^{-3} M	10	0[d]	0	0
5×10^{-2} M	100	—	—	—
Dibucaine	25	—	—	—
d-Tubocurarine	23	—p[e]	—p	—p
Quinidine sulfate	13	—	—	—

[a] Test agents were at a final conen of $10^{-3}M$ except for procaine which was also tested at a final conen of 5×10^{-2} M.

[b] + = slight stimulation.

[c] — = complete inhibition.

[d] 0 = no effect.

[e] —p = partial inhibition.

caine inhibited completely aggregation induced by thrombin, ADP, and collagen, while *d*-tubocurarine inhibited aggregation only moderately. Quinidine inhibited only slightly the ChE activity of platelets but produced complete inhibition of aggregation. None of these eight ChE inhibitors induced platelet aggregation. Acetylthiocholine iodide at the same concentration used in ChE assays increased only minimally (0—1+) the amount of aggregation of nonaged washed platelets induced by thrombin, ADP, or collagen. Platelets aged at 37° until no longer aggregable by thrombin were rendered thrombin-aggregable by addition of acetylthiocholine.

Eserine at a final concentration of 10^{-3} M produced 100% inhibition of ChE activity of the "light" fraction and 85% inhibiton of ChE activity of the "heavy" fraction of isolated platelet plasma membranes.

Comparison of ChE activity of platelets and erythrocytes. The ChE activity of intact erythrocytes was not inhibited by thrombin but was inhibited (24%) by 10^{-3} M ADP, and was slightly stimulated (9%) by collagen. The probable nonidentity of erythrocyte ChE activity with platelet ChE activity was further demonstrated by the finding that quinidine at a final concentration of 10^{-3} M completely inhibited erythrocyte ChE activity.

Discussion. It is generally accepted that two separate sites on one protein or perhaps two separate proteins on a cell membrane can react with acetylcholine (ACh). The two sites are (1) the ACh receptor site which is thought to affect membrane permeability directly and (2) the ACh esterase site which hydrolyzes ACh and thus affects permeability only indirectly. The binding of ACh to the receptor or permeability site is thought to increase membrane permeability and produce membrane depolarization (11–13) while decreased binding of ACh to the receptor site is associated with decreased permeability. Increased AChE activity could lead to depletion of ACh with decreased binding of ACh to the receptor site producing decreased permeability. The effect of ACh on the receptor site is slightly potentiated by eserine (11, 13). Hence, eserine, a specific inhibitor of AChE, would cause increased concentration and binding of ACh at the receptor site and produce increased permeability with leaking of monovalent cytoplasmic cations and perhaps other cytoplasmic components. Imipramine, FDNB, DFDNB, dibucaine, procaine, *d*-tubocurarine, and quinidine sulfate all inhibit platelet ChE activity to varying degrees. Of these agents, all except imipramine are known to react with the ACh receptor site (7, 11, 13–16) and thereby prevent

changes in membrane permeability (13). Hence, the present data can be interpreted to indicate that these ChE inhibitors, and possibly also imipramine and quinidine, inhibit platelet aggregation by their reaction with the ACh receptor site preventing the thrombin-induced or collagen-induced increase in membrane permeability and membrane depolarization. Thrombin, in addition to inducing release of certain platelet constituents including ADP, epinephrine, and potassium, is known to produce a decrease in platelet membrane surface charge (17).

It is tempting to speculate that thrombin and collagen, both inhibitors of platelet ChE even in the absence of added calcium, may induce platelet aggregation by increasing permeability of the platelet membrane through the ACh receptor site. These agents would cause increased membrane permeability with membrane depolarization and release of ADP, epinephrine, and certain other platelet constituents known to aggregate platelets directly. Thrombin-induced or collagen-induced binding of ACh to the receptor site would be augmented by the inhibition of ChE produced by these aggregating agents. Further, it appears that ACh must be available for binding to the receptor site in order for thrombin or collagen to induce platelet aggregation, for agents which inhibit ACh binding by the receptor also inhibit aggregation. The latter statement is further supported by our finding that platelets aged at 37° until they are no longer aggregable by thrombin can be rendered thrombin-aggregable again by addition of ACh.

The present study demonstrates that platelets hydrolyze 4×10^{-18} moles of ACh per platelet. Erythrocytes hydrolyze 10^{-15} moles of ACh per cell (7), but erythrocytes with an average diameter of 8 μ have approximately 100 times the surface area of platelets with an average diameter of 2.5 μ. Hence on a surface-area basis, erythrocytes have approximately 25 times the ChE activity of platelets. Platelet ChE activity appears to be a "true" cholinesterase or an acetylcholinesterase (AChE) as is the ChE of erythrocytes, since acetyl-β-methylcholine is hydrolyzed more readily than is benzylcholine.

Summary. Washed human platelets and isolated platelet-membrane fractions have been shown to have cholinesterase activity distinct from that of erythrocytes. In intact platelets the cholinesterase activity is affected by thrombin, ADP, epinephrine, or collagen. Other agents were shown to influence cholinesterase activity and seven of these also inhibited platelet aggregation induced by thrombin, ADP, or collagen. The relationship of acetylcholine and cholinesterase activity to platelet aggregation is discussed in light of these findings.

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