# Combined Effects of Succinylcholine and Calcium on Membrane-bound Acetylcholinesterase Activity

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The effects of succinylcholine and calcium ($Ca^{2+}$), alone and together, on membrane-bound acetylcholinesterase ("true-type" cholinesterase) were examined using human erythrocyte ghosts to elucidate the combined pharmacological activity of succinylcholine and calcium in in vivo system. Succinylcholine inhibited the acetylcholinesterase by a mixed style. Calcium alone exhibited an inhibitory effect on the enzyme, but a biphasic effect together with succinylcholine : marked restoration of the enzyme activity at calcium concentrations lower than 6 mM and depression at its higher concentrations. It is suggested that calcium induces a conformational change of the enzyme protein leading to the altered binding capacity of succinylcholine. In anesthetic practice, therefore, the use of calcium may not be indicated for the treatment of SCh phase II block. (Key words: succinylcholine, calcium, acetylcholinesterase, human erythrocyte membrane)

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Calcium has been shown to affect the neuromuscular blocking action of succinylcholine (SCh). Mayrhofer[1] and Irwin et al.[2] have reported that calcium decreases the duration of apnea induced by SCh in humans and dogs, respectively while Badola et al.[3] have indicated the calcium-induced depression of twitch tension during SCh desensitization block. According to Shrivastava et al.[4], recently, calcium prevented the SCh-induced myalgia. But few attempts have been made to elucidate interactions between calcium and SCh at the molecular level. Acetylcholinesterase (E.C.3.1.1.7.; AChE), which hydrolyzes and inactivates acetylcholine, plays an important role in the neuromuscular transmission. Several muscle relaxants and metal ions are thought to bind to allosteric sites on AChE and to influence its catalytic properties. Lanks and Sklar[5] have shown that SCh inhibits the activity of erythrocyte AChE in a non-competitive manner. Calcium has also been known as a modulator of AChE, and it usually activates the enzyme in solubilized preparations[6]. However, there has been no in vitro study of a combination of SCh and calcium, which may serve the pharmacological and physiological evaluation of neuromuscular transmission. It appears that membrane-bound AChE of human erythrocyte can substitute for the detergent-soluble tissue enzyme for in vitro study, though the biological function and significance of the erythrocyte AChE are unknown[7].

In the present study, we investigated the effects of SCh and calcium, alone and together, on the activity of membrane-bound AChE in human erythrocyte ghost membranes to elucidate the pharmacological interactions between

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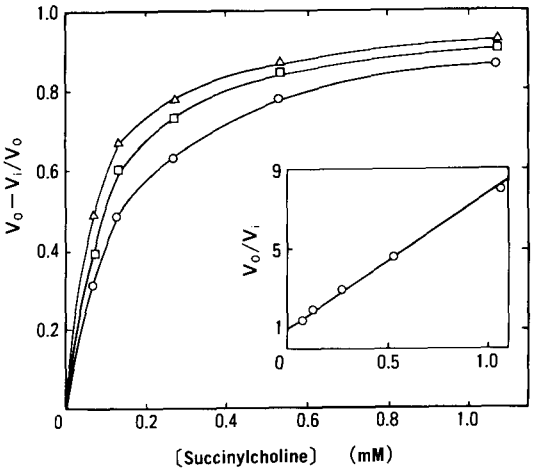


**Fig. 1.** Effect of succinylcholine on hydrolysis of acetylthiocholine. Inset shows inhibition plot of acetylcholinesterase with succinylcholine at 0.30 mM substrate. Half-maximal inactivation was observed at 0.15 mM succinylcholine. $V_i$ and $V_o$ represent the initial velocity with and without succinylcholine, respectively. Substrate concentrations were (△)0.055 mM, (□)0.11 mM and (○)0.24 mM.

SCh and calcium at the neuromuscular junction.

## Materials and Methods

Ghost membranes were prepared from human erythrocytes according to the procedure of Hanahan[8] with minor modifications. Fresh red blood cells were washed in 172 mM Tris buffer (pH 7.6) by centrifuging at 3,000 rpm for 5 min. The packed cells were hemolysed by forceful addition of 10 vol. of 11 mM Tris buffer (pH 7.6) under magnetically stirring and were followed twice by centrifugation at 10,000 rpm for 30 min at 4°C. Then the ghosts were spun down (20,000 rpm, 20 min, 4°C) and resuspended in 11 mM Tris buffer (pH 7.6). This procedure was repeated until ghost membranes became colorless. The final suspension was homogenized by a Potter-Elvehjem homogenizer (2.4 mg protein/ml), divided into 3 ml aliquots, and stored at -80°C until use. The negligible contamination of serum cholinesterase (called "pseudo-type" cholinesterase) in the ghosts was spectrophotometrically verified by the preliminary experiments with a pH indicator, bromothymol blue. Enzyme activity was assayed by the method of Ellman et al.[9] with acetylthiocholine as a substrate. The reaction mixture was composed of 5 mM 5,5′ dithiobis-(2 nitrobenzoic acid), 0.2% Triton X-100 and 100 mM inorganic phosphate in a final volume of 2 ml (pH 7.4). Instead of phosphate buffer, 12 mM Tris was used in the experiment with calcium. The reaction was initiated by adding the substrate and the increase in absorbance at 412 nm was recorded by spectrophotometer, HITACHI model 320. All kinetic measurements, at least in duplicate, were carried out at 23°C. Protein concentration was determined by the method of Lowry et al.[10] using BSA as a standard protein. The enzyme showed a Km value of $7.1 \times 10^{-5}$ M for acetylthiocholine.

Chemicals were obtained as follows : acetylthiocholine chloride from Nakarai Chemical Co. Ltd., succinylcholine chloride from Kyorin Chemical Co. Ltd. and calcium chloride from Koso Chemical Co. Ltd. All other reagents were of analytical grade commercially available. SCh was dissolved in the reaction mixture immediately before use.

## Results

*Effect of SCh on AChE activity*

SCh inhibited the AChE activity dose-dependently at low substrate concentrations (fig. 1). Inset shows the inhibition plot of AChE with SCh at 0.30 mM substrate. Half-maximal inactivation was observed at about 0.15 mM SCh. The Ki value from the Dixon plot was $6.7 \times 10^{-5}$ M (data not shown). These values agree with those observed by Lanks and Sklar[5] and by Foldes[11]. The inhibition kinetics was investigated by double reciprocal plots of AChE activity at various concentrations of SCh (fig. 2). No intersection falled on one of the axes, showing a mixed type of inhibition by SCh. SCh possesses two trimethylammonium groups like decamethonium. One of the groups could bind to the catalytic site and the other to an allosteric site, inducing conformational changes in the active center. This may explain one of the kinetics of the SCh-AChE interaction. A clinical dose of SCh, according to Foldes[11], can achieve the plasma concentration of $10^{-5}$ M order. Therefore, our result would suggest that SCh influences tissue AChE, possibly

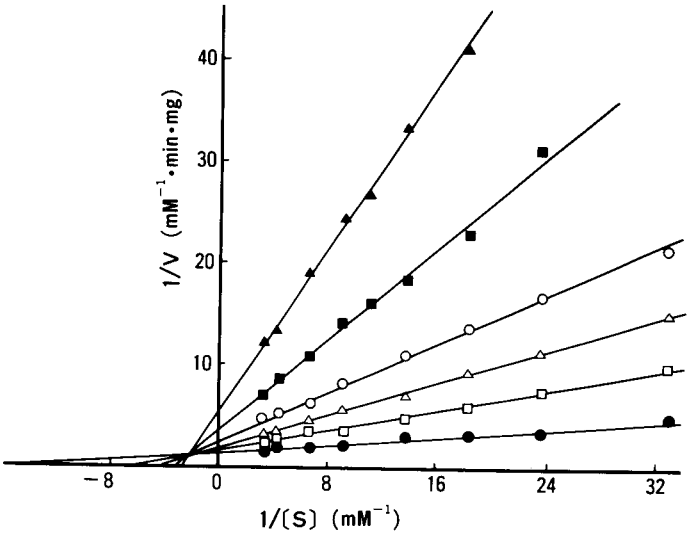


Fig. 2. Double-reciprocal plots of succinylcholine concentrations versus the rate of acetylthiocholine hydrolysis. Succinylcholine concentrations were (●)0 mM, (□)0.067 mM, (△)0.13 mM, (○)0.27 mM, (■)0.53 mM and (▲)1.07 mM.

by an allosteric mechanism, in the same concentration range as it affects cholinergic response, e.g., muscarinic effects.

*Effect of calcium on AChE activity*

Calcium suppressed the AChE activity dose-dependently (fig. 3). Inset shows the inhibition plot of AChE with calcium at 0.30 mM substrate. $ID_{50}$ of calcium was 7.1 mM. The inhibition kinetics was investigated by the Lineweaver-Burk plotting of the enzyme activity with calcium (fig. 4). Calcium also exhibited a mixed type of inhibition. Dixon plot showed a biphasic curve with a bend at about 3 mM calcium and yielded two apparent Ki (fig. 5). In 12 mM Tris buffer (pH 7.4), Ki (1) was 1.2 mM and Ki (2) 31 mM. The data were highly reproducible and independent of order in mixing. Trace amounts of calcium ion in the assay medium would not contribute to the effects observed, since no alteration in the enzyme activity was obtained by adding EDTA (0.5 mM). According to Moore and Manery[12], Triton X-100 inhibited the membrane-bound AChE even in the low concentrations. In our experiment, however, the enzyme activity was not depressed by adding 0.2% Triton X-100 to ghost suspension before the assay. Then we performed the control experiment with and without Triton X-100 to confirm that the biphasic pattern was not caused by an interaction of calcium with this detergent. In the Dixon plot was again observed the

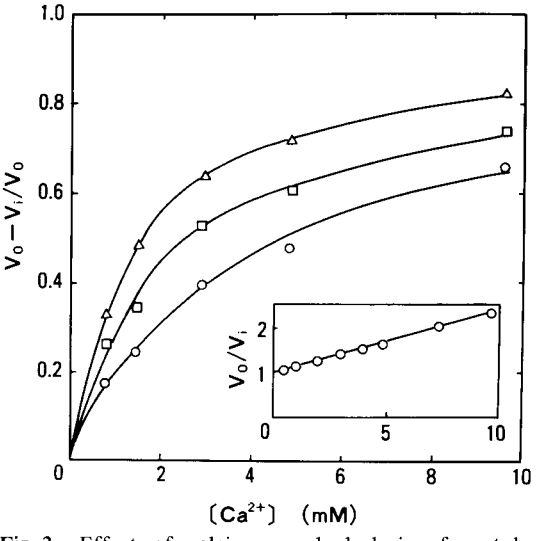


Fig. 3. Effect of calcium on hydrolysis of acetylthiocholine. Inset shows inhibition plot of acetylcholinesterase with calcium at 0.30 mM substrate. Half-maximal inactivation was observed at 7.1 mM calcium. $V_i$ and $V_0$ represent the initial velocity with and without calcium, respectively. Substrate concentrations were (△)0.030 mM, (□)0.11 mM and (○)0.24 mM.

biphasic inhibition pattern. As has been reported by Wermuth and Brodbeck[13], this biphasic nature suggests two states of membrane-bound AChE. Applying the Hill equation to the biphasic pattern at 0.030 mM substrate, the coefficient

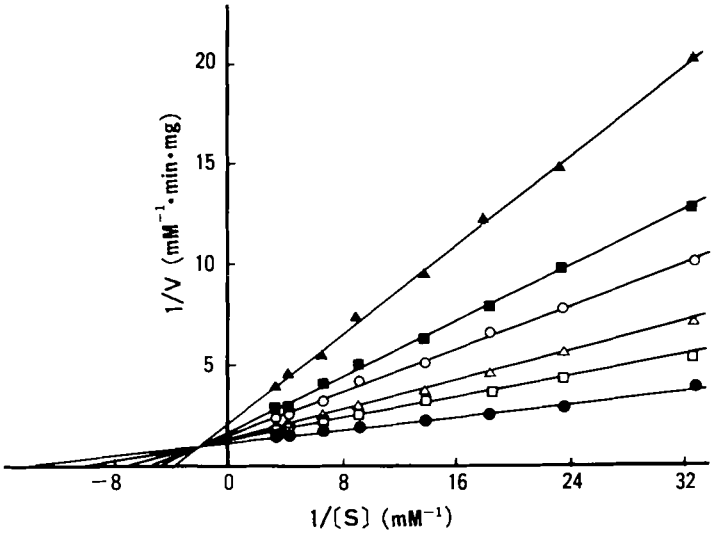


Fig. 4. Double-reciprocal plots of calcium concentrations versus the rate of acetylthiocholine hydrolysis. Calcium concentrations were (●)0mM, (□) 0.7 mM, (△)1.4 mM, (○) 2.9 mM, (■)4.8 mM and (▲)9.6 mM.

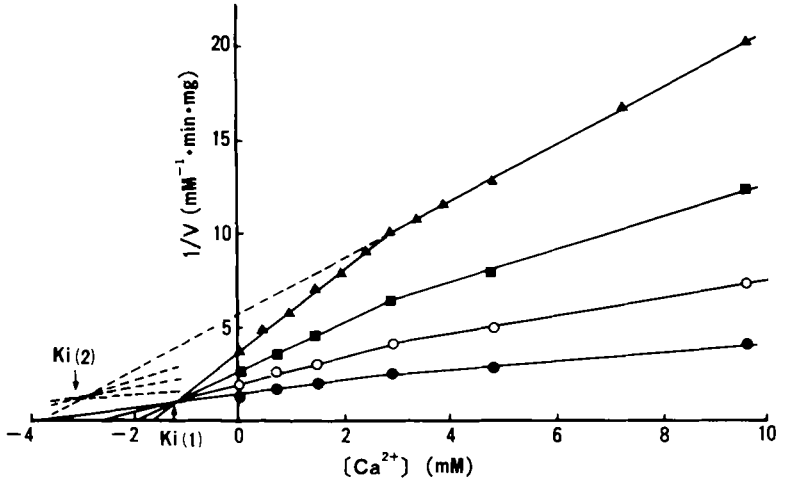


Fig. 5. Dixon plots of the effect of calcium on acetylthiocholine hydrolysis. Substrate concentrations were (▲)0.030 mM, and (■)0.54 mM, (○)0.11 mM and (●)0.30 mM.

would be 1.04 (state I) at the calcium concentration between 0 and 3.0 mM and 0.75 (state II) between 3.0 and 9.6 mM. However, based on the fact that physiological calcium concentration is lower than 2 mM, physiological significance of state II is in doubt.

*Combined effects of calcium and SCh on AChE activity*

In the presence of calcium, SCh inhibited the AChE activity, showing a hyperbolic curve in the plots (fig. 6). This may imply that SCh in combination with calcium exhibited a partially competitive kinetic for membrane-bound AChE at high SCh concentrations. At the higher calcium concentrations, the inhibition curves shifted toward higher SCh concentrations range, indicating that calcium could reverse the inhibitory effect of SCh. Calcium increased greatly the enzyme activity in the presence of SCh (fig. 7). At the higher SCh concentrations, the greater reversing activity of calcium was observed. Referring to the clinical dosage, 1.5 mM calcium could increase the enzyme activity by approximately 25% at $6.7 \times 10^{-5}$ M SCh. At calcium concentrations higher than 6 to 7 mM, however, the reversing activity was decreased.

## Discussion

In this study, calcium exerted an inhibitory effect on membrane-bound AChE of human erythrocyte, unlike the preveious observations[6,14], in which calcium served as an activator. effect on membrane-bound AChE of human erythrocyte, unlike the previous observations[6, 14], in which calcium serves as an activator. Their enzyme preparations are soluble form or mixed form of soluble and membrane-bound AChE. In general, AChE is membrane-associated and can hardly be solubilized. A review of literatures[7,15,16] has indicated that preparation and purification procedures may alter the physical properties of membrane-bound enzymes and that their catalytic functions could be restored by addition of lipids. Also, Morero et al.[17] have suggested that the membrane structure is an important factor for the allosteric changes of the membrane-bound enzymes. Thus, the discrepancy in the results may be explained by the different assay conditions. Ribaire and Kato[18] also have studied the effects of calcium on soluble and membrane-bound AChE in Electrophorus electrics, and they have found that calcium enhances the hydrolysis rate of acetylcholine by soluble AChE and decreases it by membrane-bound AChE.

When the enzyme was exposed to SCh, calcium reversed the inhibitory effect of SCh on membrane-bound AChE. Some reports have shown that calcium antagonizes the effects of other muscle relaxants on AChE activity[19,20]. Roufogalis and Quist[19] have reported that calcium reverses the inhibitory effect of decamethonium on partially purified AChE, whereas this reversal by calcium was not observed by Wins et al.[21] in thier experiment using membrane-bound AChE from the same enzyme source as that of Roufogalis and Quist. Each subunit of AChE molecule is assumed to possess minimum five anionic subsites. In the terminology of Rosenberry[22], one of them (site C) is catalytic anionic site and the others (site $P_1$ – $P_4$) are peripheral sites. Roufogalis and Quist[19] have shown that calcium antagonizes decamethonium binding to soluble AChE in a competitive manner. Tomlinson et al.[6] have suggested that decamethonium spans the C and $P_1$ site but calcium selectively binds to the

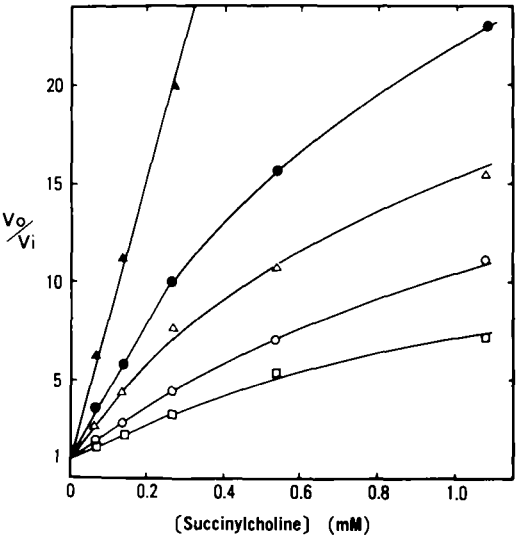


Fig. 6. Effect of succinylcholine on acetylcholinesterase activity in the presence of calcium. Calcium concentrations were (▲)0 mM, (●)1.4 mM, (^)2.8 mM, (○)5.0 mM and (□)9.6 mM. Substrate concentration was 0.30 mM. $V_i$ and $V_o$ represent the initial velocity with and without succinylcholine, respectively.

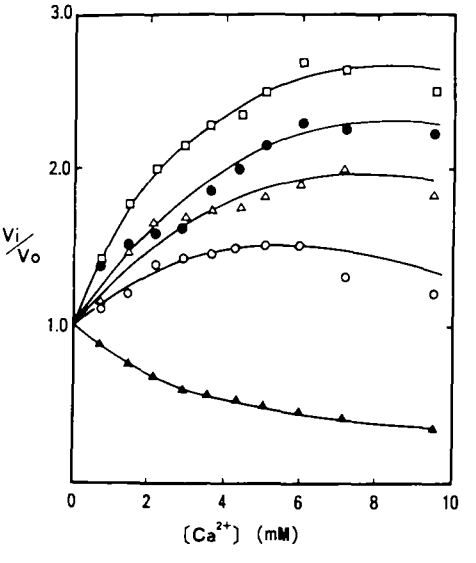


Fig. 7. Effect of calcium on the succinylcholine-induced depression of enzyme activity. Succinylcholine concentrations were (▲)0 mM, (○)0.067 mM, (^)0.13 mM, (●)0.27 mM and (□)0.54 mM. Substrate concentration was 0.30 mM. $V_i$ and $V_o$ represent the initial velocity with and without calcium, respectively.

$P_1$ site. Thus, $P_1$ site seems to function as an accelerator. In our data, however, calcium inhibited the activity of membrane-bound AChE. According to Robaire and Kato[23], moreover, membrane millieu did not significantly affect the enzymatic relationship between the peripheral site(s) and catalytic site of AChE in their study using soluble and membrane-bound enzymes. Therefore, several explanations are available for our data. The first is that calcium, only when together with SCh, would readily bind to the $P_1$ site. An alternate possibility is that the binding of calcium to allosteric sites except $P_1$ site would alter the conformation of the active center, by which SCh might lose the ability to bind to the C site and some allosteric sites.

At the neuromuscular junction, calcium enhances the release of acetylcholine from the motor nerve terminal and also stabilizes the postjunctional membranes[3]. This may explain why calcium antagonizes the neuromuscular blockade induced by magnesium or some antibiotics, especially in association with non-depolarizing muscle relaxants. However, SCh, depolarizing muscle relaxant, lacks an effective pharmacologic antagonist. In experimental animals, calcium antagonized the depolarizing block of SCh and potentiated the desensitization block, with its molecular mechanism being unknown[2,3]. The latter block, or phase II block appears to be partially reversed by cholinesterase inhibitor. The pharmacological mechanism of phase II block has not been established yet, but serveral facts seem to be involved[24]. One of them is that calcium influx through the opened ion channels can modify the physiological function of subendplate elements in muscles and of the acetylcholine receptors themselves[25] In our data calcium reversed the SCh-induced inhibition of AChE activity. Therefore, calcium will not be indicated for the treatment of phase II block. On the other hand, erythrocyte AChE itself seems to have an important role in removing the SCh administered intravenously[26]. Erythrocyte enzyme will form a SCh-AChE complex and leave less available to act at the neuromuscular junction. If so, the antagonism by calcium of SCh-induced neuromuscular blockade[1,2] may be partially explained by our present results, in which calcium reversed the inhibitory effect of SCh on erythrocyte AChE activity.

Administration of SCh is accompanied by clinically significant adverse reactions. Recently, Nigrovic[26] has interpreted adverse hemodynamic reactions to SCh by an interaction between SCh and the presynaptic cholinoceptors (nicotinic and muscarnic receptors) on the sympathetic nerve terminals. In his hypothesis, SCh usually produces balanced and clinical inconspicuous cardiovascular effects, because of the mutually opposing effects resulting from the activation of these cholinoceptors by SCh. No sufficient evidence was presented to know whether the use of calcium could keep or lose this equilibrium between nicotinic and muscarinic effects, though AChE also has a high special activity in the cholinergic system and calcium enhances the release of acetylcholine presynaptically. On the other hand, SCh-induced postoperative myalgia, usually associated with an increase in serum potassium concentration and a decrease in calcium, was prevented by calcium pretreatment[4]. Further studies must be performed to elucidate this mechanism.

In conclusion, calcium reversed the inhibitory effect of SCh on erythrocyte AChE activity. It was suggested that the use of calcium may not be indicated for the reversal of SCh phase II block, while the exact physiological and clinical significance of these observations remains to be elucidated.

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