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Activation Mechanisms of Butyrylcholinesterase by 2,4,6-Trinitrotoluene, 3,3-Dimethylbutyl-*N*-*n*-butylcarbamate, and 2-Trimethylsilyl-ethyl-*N*-*n*-butylcarbamate

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Abstract The goal of this work was to propose a possible mechanism for the butyrylcholinesterase activation by 2,4,6-trinitrotoluene (TNT), 3,3-dimethylbutyl-*N*-*n*-butylcarbamate (**1**), and 2-trimethylsilyl-ethyl-*N*-*n*-butylcarbamate (**2**). Kinetically, TNT, and compounds **1** and **2** were characterized as the nonessential activators of butyrylcholinesterase. TNT, and compounds **1** and **2** were hydrophobic compounds and were proposed to bind to the hydrophobic activator binding site, which was located outside the active site gorge of the enzyme. The conformational change from a normal active site gorge to a more accessible active site gorge of the enzyme was proposed after binding of TNT, and compounds **1** and **2** to the activator binding site of the enzyme. Therefore, TNT, and compounds **1** and **2** may act as the excess of butyrylcholine in the substrate activator for the butyrylcholinesterase catalyzed reactions.

Keywords Butyrylcholinesterase · Enzyme kinetics · Activation · TNT · Carbamate

Introduction

Butyrylcholinesterase (BChE, EC 3.1.1.8) is a serine hydrolase related to acetylcholinesterase (AChE, EC 3.1.1.7). Unlike AChE, which plays a vital role in the central and peripheral nervous systems, the physiological function of BChE remains unclear [1–4]. Despite having no identified endogenous substrate, BChE plays a key role in detoxification by degrading esters like succinylcholine and cocaine [5]. The X-ray structures of BChE and BChE–inhibitor complex have been recently reported [6, 7]. Similar to AChE [8–11], the active site of BChE contains (a) an esteratic site comprised of the catalytic triad Ser198-His438-Glu325, which is located at the bottom of the gorge [6, 7]; (b) an oxyanion hole

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composed of Gly116, Gly117, and Ala199 that stabilizes the tetrahedral intermediate; (c) an anionic substrate binding site (AS) composed of Trp82, where the quaternary ammonium pole of butyrylcholine (BCh) and of various active site ligands binds through a preferential interaction of quaternary nitrogens with the π electrons of aromatic groups; (d) an acyl group binding site that binds the acyl or carbamyl group of substrate or inhibitor; and (e) a peripheral anionic binding site composed of Phe278 [12], Tyr332 [13], and Asp70, which is located at the entrance (mouth) of the active site gorge that may bind to the tacrine-based hetero-bivalent ligands [12] and cage amines [14].

BChE-catalyzed reactions often show substrate activation at high substrate concentrations [15]. The kinetic evidence for substrate activation of BChE-catalyzed hydrolysis of butyrylthiocholine has been observed by Tormos et al. [16]. However, no other activator rather than substrates of BChE has been reported so far. Therefore, search for a substrate analog that only displays as an activator but not as a substrate of BChE will illuminate the understanding of substrate activation mechanism of the enzyme. The hydrophobic analogs of butyrylcholine, 3,3-dimethylbutyl-*N*-*n*-butylcarbamate (**1**), and 2-trimethylsilyl-ethyl-*N*-*n*-butylcarbamate (**2**) (Fig. 1) are synthesized for this purpose.

Nitro compounds such as nitroglycerin, 2,4,6-trinitrotoluene (TNT), picric acid, styphnic acid, hexahydro-1,3,5-trinitrotriazocine (RDX), and octahydro-1,3,5,7-tetranitrotriazocine (HMX; Fig. 1) are widely known as explosives [17]. Especially, TNT is hydrophobic and is slightly soluble in water. TNT has been reported as an activator of lipase [18]. Therefore, TNT is also chosen as a potential activator of BChE based on these characters.

Materials and Methods

Materials

Horse serum BChE, 5,5′-dithio-bis-2-nitrobenzoate (DTNB), and butyrylthiocholine (BTCh) were obtained from Sigma (USA). All other chemicals were of the highest purity available commercially. TNT was provided from Taiwan Army.

Synthesis of 3,3-Dimethylbutyl-*N*-*n*-butylcarbamate (**1**) and 2-trimethylsilyl-ethyl-*N*-*n*-butylcarbamate (**2**)

3,3-Dimethylbutyl-*N*-*n*-butylcarbamate (**1**) and 2-trimethylsilyl-ethyl- *N*-*n*-butylcarbamate (**2**) (Fig. 1) were synthesized from the condensation of 3,3-dimethylbutanol and 2-

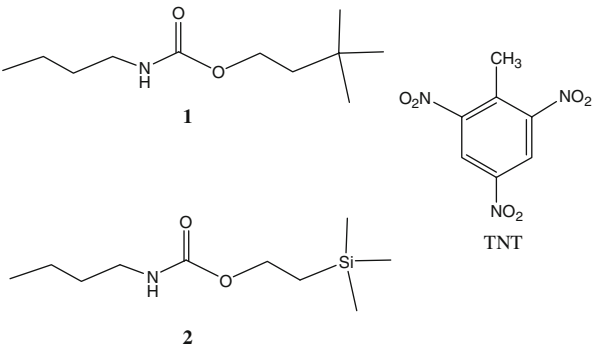


Fig. 1 Chemical structures of TNT, and compounds **1** and **2**

(trimethylsilyl)ethanol, respectively, with 1.2 equivalents of *n*-butyl isocyanate in tetrahydrofuran at 25 °C for 48 h (87% and 85% yield, respectively). The products were purified by liquid chromatography (silica gel and hexane-ethyl acetate) and characterized by ^{1}H, ^{13}C NMR, and high resolution mass spectra. 3,3-Dimethylbutyl-*N*-*n*-butylcarbamate (**1**): ^{1}H NMR ($CDCl_3$, 300 MHz) δ/ppm 0.88 (t, *J*=6 Hz, 3H, ω-CH_3), 0.89 (s, 9H, C(CH_3)$_3$), 1.30 (sextet, *J*=6 Hz, 2H, γ-CH_2), 1.44 (quintet, *J*=7 Hz, 2H, β-CH_2), 1.50 (t, *J*=7.6 Hz, 2H, CH_2C(CH$_3$)$_3$), 3.12 (m, 2H, α-CH_2), 4.07 (t, *J*=7 Hz, 2H, OCH_2CH$_2$C(CH$_3$)$_3$), and 4.72 (s, N*H*). ^{13}C NMR ($CDCl_3$, 75.4 MHz, assignment from DEPT experiments) δ/ppm 13.6 (ω-*C*H$_3$), 19.8 (γ-*C*H$_2$), 29.5 (C(*C*H$_3$)$_3$), 32.0 (β-*C*H$_2$), 40.6 (OCH$_2$CH$_2$*C*(CH$_3$)$_3$), 42.1 (α-*C*H$_2$), 63.3 (O*C*H$_2$CH$_2$C(CH$_3$)$_3$), and 156.8 (carbamate *C*=O). 2-Trimethylsilyl-ethyl-*N*-*n*-butylcarbamate (**2**): ^{1}H NMR ($CDCl_3$, 300 MHz) δ/ppm 0.11 (s, 9H, Si(CH_3)$_3$), 0.95 (t, *J*=6 Hz, 3H, ω-CH_3), 1.34 (sextet, *J*=6 Hz, 2H, γ-CH_2), 1.45 (quintet, *J*=7 Hz, 2H, β-CH_2), 1.60 (t, *J*=7.6 Hz, 2H, CH_2Si(CH$_3$)$_3$), 3.14 (m, 2H, α-CH_2), 4.12 (t, *J*=6 Hz, 2H, OCH_2CH$_2$Si(CH$_3$)$_3$), and 4.68 (s, N*H*). ^{13}C NMR ($CDCl_3$, 75.4 MHz, assignment from DEPT experiments) δ/ppm −1.5 (Si(*C*H$_3$)$_3$), 13.7 (ω-*C*H$_3$), 17.7 (OCH$_2$*C*H$_2$Si(CH$_3$)$_3$), 19.8 (γ-*C*H$_2$), 32.8 (β-*C*H$_2$), 40.7 (α-*C*H$_2$), 62.8 (O*C*H$_2$CH$_2$Si(CH$_3$)$_3$), and 156.8 (carbamate *C*=O).

Instrumental Methods

All steady state kinetic data were obtained from an ultraviolet (UV)-visible spectrophotometer (Agilent 8453) with a cell holder circulated with a water bath.

Data Reduction

Origin (version 6.0) was used for linear and nonlinear least-squares curve fittings.

Enzyme Kinetics of BChE Activation

BChE-catalyzed hydrolysis of butyrylthiocholine chloride (BTCh; 0.1 mM) in the presence of nitro compounds was followed continuously at 410 nm on a UV-visible spectrometer. The temperature was maintained at 25.0 °C by a refrigerated circulating water bath. All reactions were preformed in sodium phosphate buffer (1 ml, 0.1 M, pH 7.0) containing

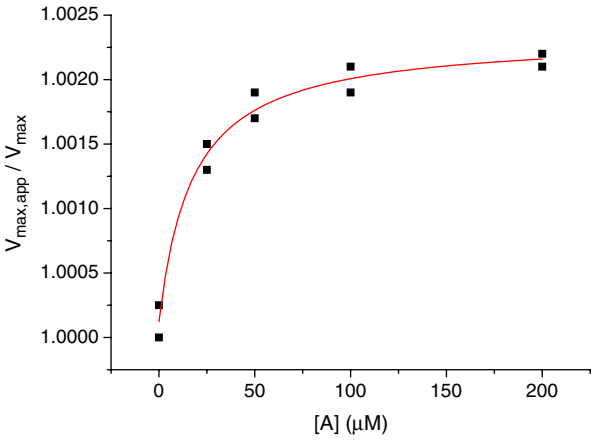


Fig. 2 Nonlinear least-squares curve fittings of the $V_{max,\ app}/V_{max}$ value for the BChE-catalyzed hydrolysis of BTCh vs. activator **1** concentration ([*A*]) plot against Eq. 2. The parameters of the fit were αK_A= 18±4 μM and β=1.0023±0.0001 (R^2=0.9815)

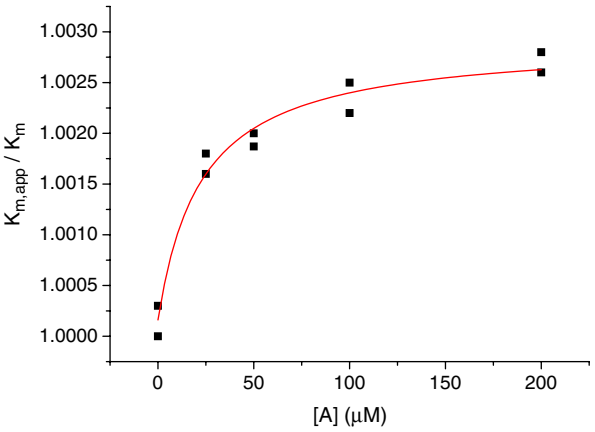


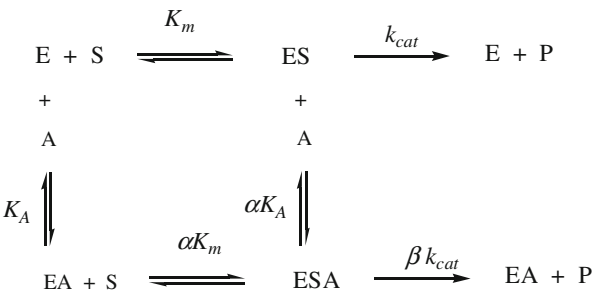
Fig. 3 Nonlinear least-squares curve fittings of the $K_{m,\ app}/K_m$ value for the BChE-catalyzed hydrolysis of BTCh vs. activator **1** concentration ([*A*]) plot against Eq. 3. The parameters of the fit were αK_A=23±6 μM and α= 1.0029±0.0002 (R^2=0.9745)

NaCl (0.1 M), CH_3CN (2% by volume), BTCh (0.0125, 0.025, 0.05, and 0.1 mM), DTNB (same concentration as BTCh) with or without an activator (compound **1** or **2**, or TNT; 0, 25, 50, 100, and 200 μM) according to Ellman's method [19]. Requisite volumes of stock solution of BTCh, DTNB, and an activator in acetonitrile were injected into reaction buffer via a pipet. BChE was dissolved in sodium phosphate buffer (0.1 M, pH 7.0). TNT and compounds **1** and **2** were characterized as the nonessential activators of the enzyme (Figs. 2 and 3, Scheme 1, and Table 1) [20]. Then, the Henri–Michaelis–Menten form the velocity equation:

$$v/V_{\max} = [S]/(K_m(1+[A]/K_A)/(1+\beta[A]/\alpha K_A) + [S](1+[A]/\alpha K_A)/(1+\beta[A]/\alpha K_A)) \quad (1)$$

In Eq. 1, $V_{\max}$ and K_m were maximal velocity and Michaelis constant in the absence of an activator, and K_A is the activation constant in the presence of an activator. The $V_{\max}$ and K_m values in the absence of an activator were 0.3223±0.005 AU/s and 110±30 μM, respectively, obtained from nonlinear least-squares curve fittings of v vs. BTCh concentration ([S]) plot against the Michaelis–Menten equation. At any activator concentration:

$$V_{\max,app}/V_{\max} = (\alpha K_A + \beta[A])/(\alpha K_A + [A]) \quad (2)$$



Scheme 1 Kinetic scheme of the nonessential activation of BChE. S, E, and A are substrate, enzyme, and activator, respectively

Table 1 The K_A, α, and β values for the BChE activation by TNT, and compounds **1** and **2**.

Compounds	K_A (μM)	α[a]	β[b]
1	21±8	1.0029±0.0002	1.0023±0.0001
2	32±8	1.0060±0.0007	1.0052±0.0004
TNT	100±30	1.008±0.003	1.007±0.002

[a] Obtained from the nonlinear least-squares curve fittings of the $K_{m,\ app}/K_m$ values vs. [A] plot against Eq. 3 (Fig. 3)

[b] Obtained from the nonlinear least-squares curve fittings of the $V_{max,\ app}/V_{max}$ values and vs. [A] plot against Eq. 2 (Fig. 2)

$$K_{m,\,app}/K_m = (\alpha K_A + \alpha[A])/(\alpha K_A + [A]) \tag{3}$$

In Eqs. 2 and 3, $V_{max,\ app}$ and $K_{m,\ app}$ were apparent maximal velocity and apparent Michaelis constant in the presence of an activator. The αK_A and β values were then obtained from the nonlinear least-squares curve fittings of the $V_{max,\ app}/V_{max}$ values vs. activator concentration ([A]) plot against Eq. 2 (Fig. 2). The αK_A and α values were then obtained from the nonlinear least-squares curve fittings of the $K_{m,\ app}/K_m$ values vs. activator concentration ([A]) plot against Eq. 3 (Fig. 3). Thus, the K_A values could be calculated from the αK_A and α values (Table 1). Duplicate sets of data were collected for each activator concentration.

Stop-time Assay for BChE Activation

For the control experiment (with an activator without incubation), BChE was incubated alone for a period of time (t) at 25.0 °C before starting the reaction by addition of the substrate BTCh (0.1 mM), DTNB (0.1 mM), and an activator (0.1 mM) to the enzyme solution (Fig. 4). For the incubation of BChE with an activator experiment, BChE was incubated with an activator (0.1 mM) for a period of time (t) at 25.0 °C before starting the reaction by addition of the substrate BTCh (0.1 mM) and DTNB (0.1 mM) to the incubated mixture. For the incubation of BChE with an activator and an inhibitor (edrophonium)

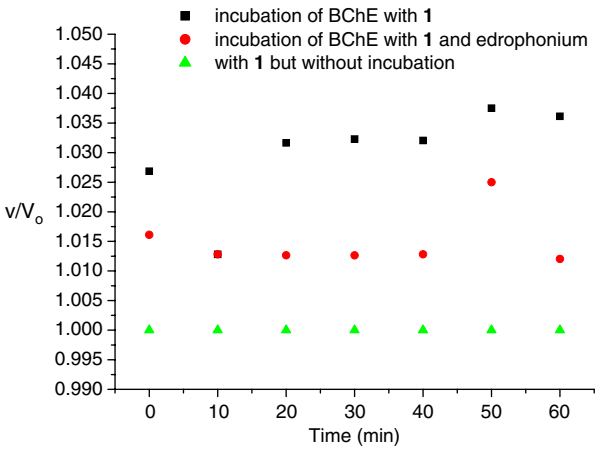


Fig. 4 Stop-time assay for BChE-catalyzed hydrolysis of BTCh in the presence of activator **1** with or without incubation of the enzyme with activator **1** or both activator **1** and edrophonium. [BTCh]=[**1**]=[edrophonium]=0.1 mM. V_0 was the initial velocity for the BChE-catalyzed hydrolysis of BTCh, and v was the observed initial velocity at any condition

experiment, BChE was incubated with an activator (0.1 mM) and edrophonium (0.1 mM) for a period of time (t) at 25.0 °C before starting the reaction by addition of the substrate BTCh (0.1 mM) and DTNB (0.1 mM) to the incubated mixture. The initial velocity (v) of the reaction was calculated from the time course. All the other procedures were similar to those described in the *Enzyme kinetics of BChE activation* part.

Results

TNT, and compounds **1** and **2** are characterized as the nonessential activators [20] of BChE (Figs. 2 and 3, Scheme 1, Table 1, and Eq. 1). Thus, the dissociation constant for activation (or activation constant), αK_A and the α value are determined from nonlinear least-squares

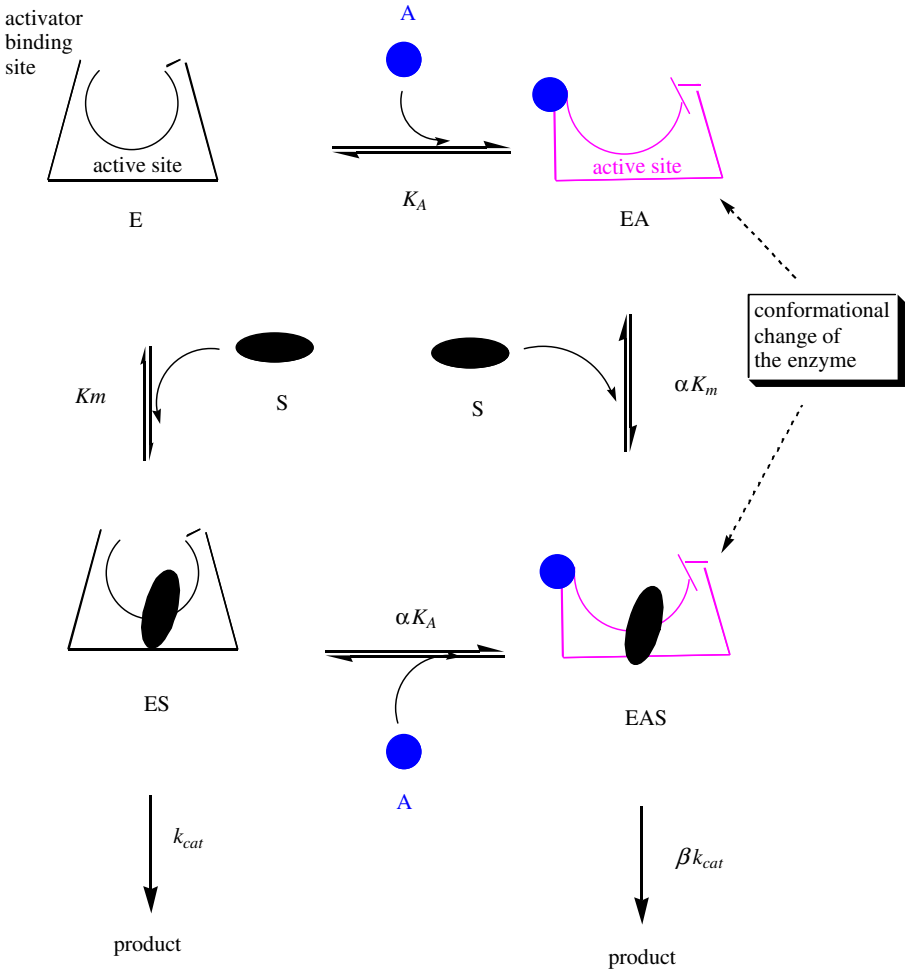


Fig. 5 Schematic representation of the nonessential activation of BChE by TNT, compounds **1** or **2** in the presence of substrate, BTCh. The notation is the same with that of Scheme 1. TNT, and compounds **1** and **2** may induce a conformational change of the enzyme. The activator may bind to the activator binding site of the enzyme, which is located outside the active site like the co-lipase binding site to lipase [19, 20]

curve fittings of the plot of the $V_{max,\ app}/V_{max}$ values on [A] against Eq. 2 (Fig. 2 and Table 1). On the other hand, αK_A and the β value can be determined from nonlinear least-squares curve fittings of the plot of the $K_{m,\ app}/K_m$ values on [A] against Eq. 3 (Fig. 3 and Table 1). That all β and α values are slightly more than one (Table 1) indicates that TNT, and compounds **1** and **2** are characterized as the nonessential activators of BChE. From the stop-time assay, TNT, and compounds **1** and **2** are confirmed as activators of BChE (Fig. 4).

Discussion

Since TNT, and compounds **1** and **2** are characterized as the nonessential activators of BChE (Table 1), TNT, and compounds **1** and **2**may bind outside the active site gorge of BChE like the co-lipase binding site of lipase [21, 22] (Fig. 5). Then, TNT, and compounds **1** and **2** may induce a conformational change of BChE and activate the reaction (Fig. 5). Thus, the substrate activation of BChE may act like the nonessential activator of the enzyme by binding extra substrate outside the active site of the enzyme and activating the enzyme.

The K_A value for BChE activation by ATCh has been calculated to be 3.5 mM [16]. In this study, the K_A values for BChE activations by TNT, and compounds **1** and **2** are calculated to be 100, 21, and 32 μM, respectively (Figs. 2 and 3, and Table 1). Therefore, TNT, and compounds **1** and **2** are much potent activators than ATCh. On the other hand, the β value of 2.6 has been reported for the BChE activation by ATCh [16], which is much higher than the β values from this study.

Structurally, the hydrophilic active site of BChE is too hydrophilic to accept hydrophobic compounds like TNT, and compounds **1** and **2**. On the other hand, the activator binding site of BChE is outside the active site gorge and is similar to the catalytic site in size (Fig. 4) since huge, bulky compounds such as cage compounds [14], RDX, and HMX are not activators of the enzyme (data not shown). At present, we find that only small, hydrophobic molecules such as TNT, and compounds **1** and **2** are the non-essential activators of BChE.

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