Potentiating Effects of β-Eudesmol-Related Cyclohexylidene Derivatives on Succinylcholine-Induced Neuromuscular Block in Isolated Phrenic Nerve-Diaphragm Muscles of Normal and Alloxan-Diabetic Mice

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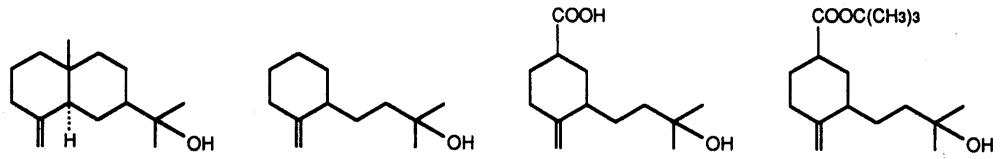
β-Eudesmol, a sesquiterpenoid alcohol contained in *Atractylodes lancea*, potentiates succinylcholine (SuCh)-induced neuromuscular blockade. The potentiating effect is greater in diabetic muscles than in normal ones. As a ligand for affinity chromatography to study the potentiating mechanism, we designed and synthesized newly β-eudesmol-related cyclohexylidene derivatives (2-(3-hydroxy-3-methylbutyl)cyclohexylidene; KTE-13, 2-(3-hydroxy-3-methylbutyl)-4-cyclohexylidene carboxylic acid; KTE-32 and 4-*tert*-butoxycarbonyl-2-(3-hydroxy-3-methylbutyl) cyclohexylidene; KTE-33). We examined the potentiating effects of those compounds in phrenic nerve-diaphragm muscle preparations of normal and alloxan-diabetic mice. KTE-33 (100 μM) potentiated more greatly SuCh-induced neuromuscular blockade in diabetic muscles than in normal ones (the potentiating ratios in normal and diabetic muscles were 6.7 and 10.6, respectively), while KTE-13 (100 μM) and -32 (200 μM) potentiated weakly. These results suggest that the ester group in KTE-33 rather than a carboxyl group in KTE-32 is important in inducing the potentiation of SuCh-induced neuromuscular blockade in diabetic state.

Key words β-eudesmol-related compound; cyclohexylidene derivative; neuromuscular blockade; succinylcholine; potentiation; diabetic mouse

β-Eudesmol is a sesquiterpenoid alcohol contained in *Atractylodes lancea*, one of the constituents of prescribed medicines which have been used in traditional Japanese-Sino medicine to alleviate muscular pain.[1] We previously reported that β-eudesmol potentiates the neuromuscular blocking effect of succinylcholine (SuCh) and that the potentiating effect is greater in diabetic muscles than in normal ones.[2] The mechanism of potentiation by β-eudesmol in both types of muscles is not yet clear. Thus, as a ligand for affinity chromatography to study the potentiating mechanism, we designed and synthesized β-eudesmol-related compounds (2-(3-hydroxy-3-methylbutyl)-cyclohexylidene; KTE-13, 2-(3-hydroxy-3-methylbutyl)-4-cyclohexylidene carboxylic acid; KTE-32 and 4-*tert*-butoxycarbonyl-2-(3-hydroxy-3-methylbutyl)cyclohexylidene; KTE-33) (Fig. 1). The chemical structure of KTE-13 is partly the same as that of β-eudesmol which is essential for its potentiating effect on SuCh-induced neuromuscular blockade.[3] The compound, KTE-32, has a carboxyl group which is introduced to the cyclohexane ring of KTE-13. We also synthesized an esterified compound (KTE-33) of KTE-32, which may be suitable since an immobilized form existed in affinity gel. In this study, we examined the potentiating effects of those compounds on SuCh-induced neuromuscular blockade in phrenic nerve-diaphragm muscle preparations of normal and alloxan-diabetic mice.

MATERIALS AND METHODS

Potentiation of SuCh-Induced Neuromuscular Block Male mice (ddY strain, 4 weeks old) were injected with alloxan monohydrate (85 mg/kg, Nacalai Tesque, Kyoto) into a tail vein and were used 4—5 weeks later. The phrenic nerve-diaphragm muscles of normal (30—41 g) and alloxan-diabetic (24—39 g, feeding blood glucose level: 300—600 mg/dl) mice were isolated and suspended in 5 ml of Krebs-Henseleit solution (137 mM NaCl, 5.0 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 15 mM $NaHCO_3$ and 10 mM glucose) bubbled with 95% O_2 +5% CO_2 at 35±2 °C. The nerve and muscle were stimulated alternately at a rate of 0.2 Hz through a pair of platinum electrodes (1 ms duration and supramaximal voltage). The muscle tension was recorded isometrically under 1 g loading tension. SuCh was applied cumulatively at 2-min intervals after pretreatment with each cyclohexylidene derivative for 60 min. The inhibitory responses were represented as a percentage of the control value for 1 min



β-eudesmol KTE-13 KTE-32 KTE-33

Fig. 1. Chemical Structures of β-Eudesmol and Its Related Compounds

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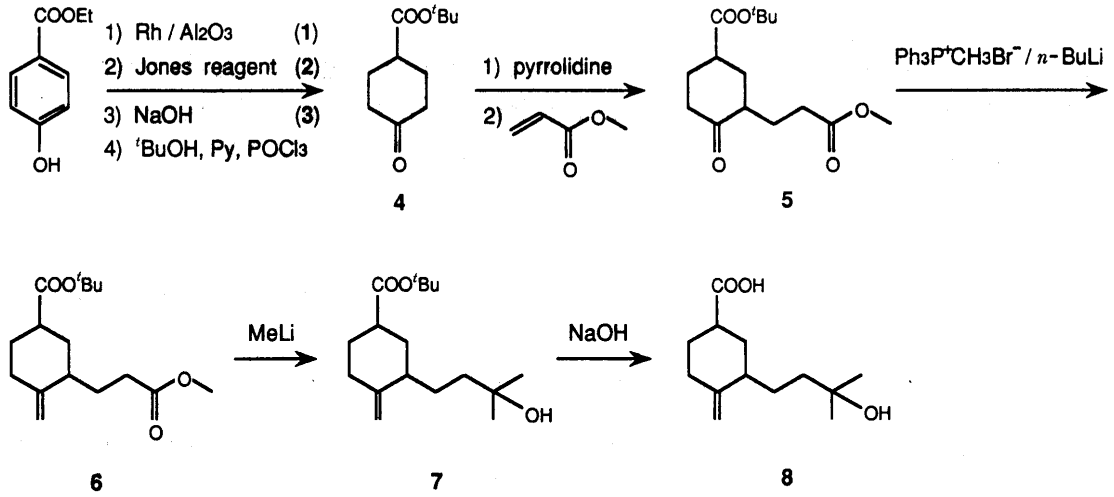

Chart 1. Synthesis of KTE-32 and KTE-33

Compounds **7** and **8** are KTE-33 and KTE-32, respectively.

before applying SuCh. All compounds were dissolved in 0.2% (v/v) propylene glycol. This concentration of propylene glycol did not affect the twitch response. The control response of SuCh was obtained after pretreatment with 0.2% (v/v) propylene glycol. The potentiating ratio was taken as the ratio of the 50% inhibitory concentration (IC_{50}) of SuCh obtained without to that with cyclohexylidene derivatives.

Chemistry KTE-13 was synthesized as described in ref. 3. Elemental analyses were performed on a Yanako MT-5 elemental analyzer. ^{1}H-NMR spectra were recorded on either a Jeol FX 90Q or Jeol JNM-EX270 spectrometer. The chemical shifts were given as ppm values with tetramethylsilane as an internal standard. KTE-32 and KTE-33 were synthesized as follows (Chart 1).

Ethyl 4-Hydroxycyclohexane Carboxylate (**1**): Ethyl 4-hydroxybenzoate (50 g, 300 mmol) with 5% Rh/Al_2O_3 (1 g) in ethanol (100 ml) was hydrogenated at room temperature under 5 atm for 20 h. After the product was filtered and evaporated, 57 g (100%) of **1** was obtained.

4-Ethoxycarbonylcyclohexanone (**2**): Jones reagents (8 N) was added to a solution of **1** (57 g, 300 mmol) in acetone (200 ml). The reaction mixture was poured into water, extracted with CH_2Cl_2, washed (brine), dried ($MgSO_4$) and evaporated to give 66.2 g (100%) of **2** as a colorless oil.

4-Cyclohexanone Carboxylic Acid (**3**): A solution of **2** (20 g, 117 mmol) and NaOH (10 g) in 90% ethanol (100 ml) was stirred at room temperature for 5 h. The reaction mixture was poured into saturated NH_4Cl solution, extracted with $CHCl_3$, washed (brine), dried ($MgSO_4$) and evaporated to give 13.4 g (80%) of **3** as a colorless oil.

4-*tert*-Butoxycarbonylcyclohexanone (**4**): Phosphorus oxychloride (10.5 ml, 114 mmol) was added to a solution of **3** (13.4 g, 78 mmol), *tert*-butylalcohol (72 ml) and pyridine (50 ml) at 0 °C. The reaction mixture was stirred at room temperature for 4 h, and then it was poured into water, extracted with ethyl acetate, washed (2 N HCl), dried ($MgSO_4$) and evaporated. After purification by column chromatography, 12.2 g (80%) of **4** was obtained as a colorless oil.

4-*tert*-Butoxycarbonyl-2-(2-methoxycarbonylethyl)-cyclohexanone (**5**): A solution of **4** (12.2 g, 61 mmol) and pyrrolidine (6 ml) in benzene (200 ml) was refluxed for 12 h. The reaction mixture was evaporated under reduced pressure, and then it was added to a solution of methylacrylate (11 ml) in benzene (200 ml). The reaction mixture was refluxed for 4 h, and then was stirred in water at room temperature for 2 h, extracted with ethyl acetate, washed (dil. HCl), dried ($MgSO_4$) and evaporated. After purification by column chromatography, 11.4 g (65%) of **5** was obtained as a colorless oil.

4-*tert*-Butoxycarbonyl-2-(2-methoxycarbonylethyl)-cyclohexylidene (**6**): A solution of 1.6 M *n*-butyllithium (30 ml) in hexane was added to a solution of methyltriphenylphosphonium bromide (16 g) in tetrahydrofuran (THF, 100 ml). The reaction mixture was added to a solution of **5** (11.4 g, 40 mmol) in THF (20 ml), and then it was stirred at room temperature for 12 h. The reaction mixture was poured into water, extracted with ether, washed (brine), dried ($MgSO_4$) and evaporated. After purification by column chromatography, 4.2 g (37%) of **6** was obtained as a colorless oil.

4-*tert*-Butoxycarbonyl-2-(3-hydroxy-3-methylbutyl)-cyclohexylidene (**7**; KTE-33): A solution of 1.06 M methyllithium (30 ml) in ether (30 ml) was added dropwise to a solution of **6** (4.2 g, 15 mmol) in THF (50 ml) at −78 °C. The reaction mixture was stirred at room temperature, and then was poured into brine, extracted with $CHCl_3$, washed (brine), dried ($MgSO_4$) and evaporated. After purification by column chromatography, 3.6 g (86%) of **7** was obtained as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ : 1.28—1.99 (9H, m), 2.10—2.33 (3H, m), 2.46—2.62 (1H, m), 4.62 (1H, d, J=1.49 Hz), 4.68 (1H, d, J=1.49 Hz). *Anal.* Calcd for $C_{17}H_{30}O_3$: C, 72.30; H, 10.71. Found: C, 70.91; H, 11.08.

2-(3-Hydroxy-3-methylbutyl)-4-cyclohexylidene Carboxylic Acid (**8**; KTE-32): A solution of **7** (2.2 g, 8 mmol) and NaOH (10 g) in 80% ethanol (180 ml) was refluxed for 12 h. The reaction mixture was neutralized with NH_4Cl, extracted with $CHCl_3$, washed (brine), dried ($MgSO_4$) and evaporated. After purification by column chromatography

with silica gel and ODS-silica gel, 0.8 g (45%) of **8** was obtained as a mixture of diastereomers. ^{1}H-NMR ($CDCl_3$) δ: 1.22 (9H, s), 1.24 (9H, s), 1.32—2.77 (12H, m), 4.61—4.79 (2H, m), 6.16 (1H, br s). *Anal.* Calcd for $C_{13}H_{22}O_3$: C, 68.99; H, 9.80. Found: C, 68.67; H, 9.92.

RESULTS AND DISCUSSION

SuCh blocked nerve-evoked twitch responses concentration-dependently in the isolated phrenic nerve-diaphragm muscle preparations of normal and diabetic mice. The blocking effect of SuCh was greater in diabetic muscle than in normal one (Fig. 2). The potency ratio was 1.41. This result was comparable to that reported previously.[2] Each KTE-13 (80 and 100 μM), -32 (80—200 μM) and -33 (80 and 100 μM) itself did not affect the twitch response. KTE-13 does not have a functional group available for affinity chromatography in the chemical structure. This compound showed a potentiating effect at 100 μM, weaker than that of β-eudesmol (Table I). KTE-32, in which a carboxyl group is introduced to the cyclohexane ring of KTE-13, had a weakly potentiating effect at the concentration of 200 μM in diabetic muscle (Fig. 2 and Table I). KTE-33, which is a *tert*-butyl ester of KTE-32, greatly potentiated the SuCh-induced neuromuscular blockade at 100 μM in both normal and diabetic muscle preparations, with the effect greater in diabetic muscle (Fig. 3 and Table I).

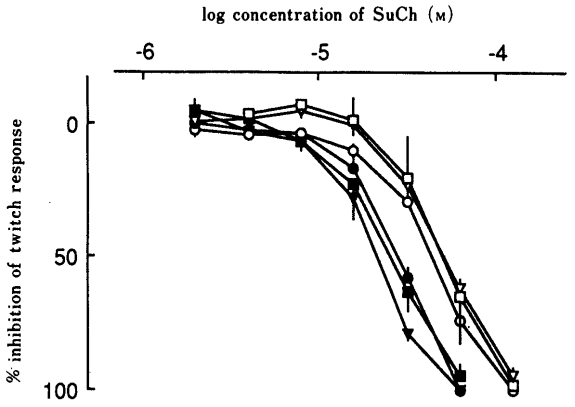

Fig. 2. Potentiating Effects of KTE-32 on SuCh-Induced Neuromuscular Blockade in Phrenic-Nerve Diaphragm Muscles of Mice

The percent inhibitions of the twitch response in normal (open symbols) and alloxan-diabetic (closed symbols) muscles pretreated without (○; ●) and with KTE-32 (□; ■: 100 μM, ▽; ▼: 200 μM) for 1 h are plotted against the log concentration of SuCh. IC_{50} (95% confidence limit, μM): 41.3 (39.0—43.8, ○), 49.4 (26.2—94.2, □), 53.4 (47.5—60.1, ▽), 29.3 (22.9—37.4, ●), 25.6 (20.5—31.9, ■) and 20.1 (17.1—23.7, ▼). Values are mean±S.E.M. (n=3—5). IC_{50} ratios of SuCh without to with KTE-32 are summarized in Table I.

TABLE I. Potentiating Effects of β-Eudesmol-Related Cyclohexylidene Derivatives (KTE-13, -32 and -33) on SuCh-Induced Neuromuscular Block in Phrenic Nerve-Diaphragm Muscle Preparations of Normal and Alloxan-Diabetic Mice

	μM	IC_{50} ratio[a]	
		Normal	Alloxan-diabetic
KTE-13	80	1.15 (4)[c]	N.T.
	100	2.20 (4)	N.T.
KTE-32	80	0.95 (3)	N.T.
	100	0.84 (4)	1.14 (3)
	200	0.77 (4)	1.46 (3)
KTE-33	80	1.10 (4)	2.03 (5)
	100	6.74 (5)	10.6 (4)
β-Eudesmol[b]	80	2.69	6.31

a) IC_{50} ratio of SuCh without/with cyclohexylidene derivative. *b*) Data were taken from ref. 2. *c*) The number of observations is in parenthesis. N.T.: not tested.

β-Eudesmol itself does not affect neuromuscular transmission at below 80 μM, but blocks in high concentration and then decreases endplate potential without affecting quantal content.[4] Single channel recordings indicate that β-eudesmol blocks the nicotinic acetylcholine receptor-channels in normal skeletal muscle cells.[4] The potentiating effect of β-eudesmol is probably due to postsynaptically accelerated desensitization of nicotinic receptors.[5] In the present study, KTE-33 showed a greater potentiating effect in diabetic than in normal muscle. These effects of KTE-33 were entirely different from those of KTE-32. The potentiating pattern of KTE-33 in normal and diabetic muscles was the same as that of β-eudesmol although its potency at 80 μM was low compared with that of β-eudesmol in both muscles. These results indicate that KTE-33 modifies the neuromuscular blocking effect of SuCh and its effect becomes hypersensitive, especially in diabetic muscles, because KTE-33 does not cause a neuromuscular blockade by itself.

SuCh with KTE-33 blocked the nerve-evoked twitch response but not the directly stimulated contraction in the isolated phrenic nerve-diaphragm muscle preparations of normal and diabetic mice. The blocking effect was readily reversed by washout. Neostigmine (15 μM), a cholinesterase inhibitor fully reversed the complete blockade caused by 6.5 μM (+)-tubocurarine, but not that caused by SuCh with KTE-33 (data not shown). These results suggested that the potentiation by KTE-33 of SuCh-induced blocking is caused neither by the change of susceptibilities to the voltage-dependent Na channel nor the intramuscular contractile system in diabetic state. We have reported that the hypersensitivity of the presynaptic nicotinic receptor to SuCh is produced in the phrenic nerve

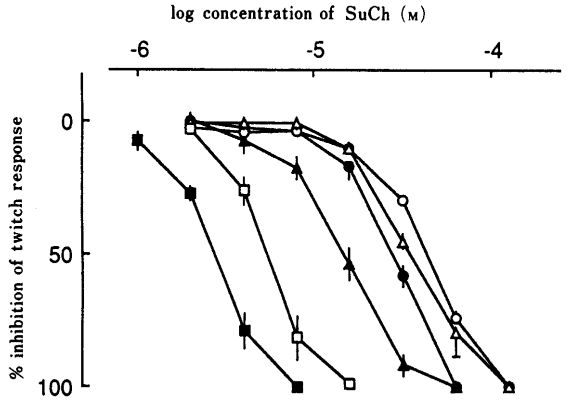

Fig. 3. Potentiating Effects of KTE-33 on SuCh-Induced Neuromuscular Blockade in Phrenic-Nerve Diaphragm Muscles of Mice

The percent inhibitions of the twitch response in normal (open symbols) and alloxan-diabetic (closed symbols) muscles pretreated without (○; ●) and with KTE-33 (△; ▲: 80 μM, □; ■: 100 μM) for 1 h are plotted against the log concentration of SuCh. IC_{50} (95% confidence limit, μM): 41.3 (39.0—43.8, ○), 37.5 (31.6—44.3, △), 6.13 (4.94—7.62, □), 29.3 (22.9—37.4, ●), 14.4 (10.8—19.2, ▲) and 2.77 (2.45—3.13, ■). Values are mean±S.E.M. (n=4—5). IC_{50} ratios of SuCh without to with KTE-33 are summarized in Table I.

of streptozocin-diabetic mice.[6] Some nicotinic channel blockers, including β-eudesmol are thought to accelerate the agonist-induced desensitization of its receptors.[4,7,8] Hence the potentiating effect of KTE-33 on SuCh-induced blocking may also be caused by accelerated desensitization of the pre- and post-synaptic nicotinic receptors.

KTE-33, an esterified compound of KTE-32, may be an immobilized form of KTE-32 in an affinity gel. KTE-32 may therefore be a prototype of ligand for affinity chromatography to study the potentiating mechanism of SuCh-induced neuromuscular blockade in diabetic state.

In conclusion, the ester group in KTE-33 rather than a carboxyl group in KTE-32 is important in inducing the potentiation of SuCh-induced neuromuscular blockade in diabetic state.

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