Pharmacological Studies of the Crude Methanol Extract from *Piper sarmentosum* Roxb. on the Neuromuscular Junction in Rats

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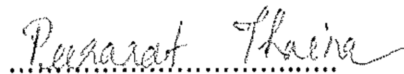
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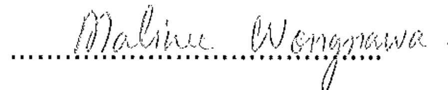
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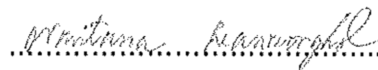
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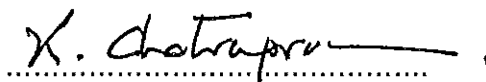
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ชื่อวิทยานิพนธ์	การศึกษาฤทธิ์ทางเภสัชวิทยาของสารสกัดหยาบจากชะพลูด้วยเมธานอลต่อบริเวณรอยต่อปลายประสาทกล้ามเนื้อลายในหนูขาว
ผู้เขียน	นายวัฒนา รัตนพรหม
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บทคัดย่อ

การวิจัยนี้เป็นการศึกษาฤทธิ์ในการคลายกล้ามเนื้อของสารสกัดจากใบชะพลูด้วยเมธานอลนอกร่างกาย โดยใช้กล้ามเนื้อกระบังลมพร้อมเส้นประสาทฟรีนิกของหนูขาว จากการศึกษาพบว่าสารสกัดในความเข้มข้นต่ำ (1.6 และ 3.2 มก/มล) ทำให้แรงหดตัวของกล้ามเนื้อเพิ่มขึ้น เมื่อกระตุ้นด้วยไฟฟ้าผ่านทางเส้นประสาทและกระตุ้นที่กล้ามเนื้อโดยตรง ในขณะที่สารสกัดในความเข้มข้นสูงขึ้น (4.0, 4.8 และ 6.4 มก/มล) แรงหดตัวของกล้ามเนื้อเพิ่มขึ้นในช่วงเวลาสั้นๆ แล้วตามด้วยการคลายกล้ามเนื้อโดยที่สารสกัดไม่มีผลต่อการนำสัญญาณประสาท ฤทธิ์ในการคลายกล้ามเนื้อมีความสัมพันธ์โดยตรงกับความเข้มข้นที่เพิ่มขึ้น ซึ่งผลในการคลายกล้ามเนื้อของสารสกัด เมื่อกระตุ้นด้วยไฟฟ้าผ่านทางเส้นประสาทจะเด่นชัดกว่าการกระตุ้นที่กล้ามเนื้อโดยตรง

เมื่อเปรียบเทียบฤทธิ์ในการคลายกล้ามเนื้อของสารสกัดกับ d-tubocurarine (dTC) และ succinylcholine (SCh) พบว่ามีค่า EC_{50} เท่ากับ 4.07 มก/มล, 1.1 ไมโครโมล่าร์ และ 15 ไมโครโมล่าร์ ตามลำดับ สารสกัดสามารถเสริมฤทธิ์ของ dTC และ SCh ส่วน tetraethylammonium (TEA)

สามารถต้านฤทธิ์ของสารสกัดได้ชั่วคราวเท่านั้น ในขณะที่ neostigmine ไม่สามารถต้านฤทธิ์ของสารสกัดได้ นอกจากนั้นสารสกัดสามารถยับยั้งการหดตัวของกล้ามเนื้อที่เกิดจากการใช้ acetylcholine ในขนาดสูง ดังนั้นจากการศึกษาครั้งนี้แสดงให้เห็นว่าฤทธิ์ในการคลายกล้ามเนื้อของสารสกัดที่เกิดขึ้นนั้นเกิดที่บริเวณรอยต่อประสาทกล้ามเนื้อลาย (neuromuscular junction) และน่าจะเป็นผลมาจาก การยับยั้งการหลั่งสารสื่อประสาทออกจากปลายประสาทก่อนซินแนป (presynaptic terminals) และมีการปิดกั้นที่รีเซพเตอร์หลังซินแนป (postsynaptic receptors) ร่วมด้วย

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ABSTRACT

The crude methanol extract (ME) of *Piper sarmentosum* Roxb. (Piperaceae) was studied in rat phrenic nerve-hemidiaphragm preparation. The ME produced only twitch potentiation at lower concentrations (1.6 and 3.2 mg/ml) but at higher concentrations (4.0, 4.8 and 6.4 mg/ml) it caused transient twitch potentiation then followed by twitch depression in both neurally- and directly-evoked twitch without depressive effect on nerve action potential conduction. The ME exhibited a marked dose-related neurally-evoked twitch depression.

The neuromuscular blocking effect produced by the extract was compared to reference drugs, d-tubocurarine (dTC) and succinylcholine (SCh). The EC_{50} for neurally-evoked twitch depression of ME, dTC, SCh were 4.07 mg/ml, 1.1 μM and 15 μM, respectively. At low concentrations, ME.significantly antagonized the depressive effect

of dTC, while at high concentrations, it synergized depressive effect on the preparation in the presence of dTC. All concentrations of ME produced only synergistic effect on the preparation in the presence of SCh. However, it was transiently and partially antagonized by tetraethylammonium (TEA) but not by neostigmine (NS). Furthermore, the extract could suppress the twitch amplitude of acetylcholine (ACh) induced contraction.

These findings suggested that the extract possessed a marked neuromuscular blocking activity at neuromuscular junction (NMJ) and the possible sites of mechanisms were likely to involve in the inhibition of neurotransmitter (acetylcholine) release at presynaptic terminals and the blocking of postsynaptic nicotinic receptors.

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LIST OF ABBREVIATIONS

ACh	=	acetylcholine
AChR	=	acetylcholine receptor
AChE	=	acetylcholinesterase
NMJ	=	neuromuscular junction
ME	=	methanol extract of *Piper sarmentosum* Roxb.
kg	=	Kilogram
Hz	=	hertz
mM	=	millimolar
ml	=	millilitre
ms	=	millisecond
mV	=	millivolt
mg	=	milligram
μM	=	micromolar
°C	=	degree Celsius
%	=	percent
xxx™	=	trade mark
®	=	trade name
EC_{50}	=	effective concentration at 50% of maximum response
P	=	*P* value
r	=	correlation coefficient

LIST OF ABBREVIATIONS (CONT.)

rpm	round per minute
SE	standard error
g	gram
No.	number
w/w	weight by weight

CHAPTER 1

INTRODUCTION

In the recent years, the medical sciences are in a rapid progression. New technologies and modern medicines are used in intervention for several diseases. Today, however, the Thai traditional medicines are not looked over. The Royal Thai government has seen in the importance of self-reliance on drug supplies of the people who live in remote areas where are difficult to obtain modern medicines, and attempted to integrate the traditional and modern systems of medicine to apply them properly. By the Ministry of Public Health, medicinal plants had being promoted through the primary health care system to encourage self-reliance on drug supplies. In fact, medicinal plants have played an important role in the medical system of Thai people in the past, they were used in intervention for several diseases for a long time as evidences seen in the several Thai traditional medicine textbooks, but they were not confirmed in effectiveness and safety by scientific processes.

Several kinds of medicinal plants in Thailand were scientifically tested and exhibited their pharmacological activities according to traditional medicine textbooks. Later, they were

developed to commercial preparations or raw materials in many pharmaceutical industries.

In pharmacological studies, natural products can exert their different pharmacological activities on different target organs. Among these, the neuromuscular junction is one of the interesting target sites for studying in pharmacological activities of natural products. Many natural products can exert their activities on neuromuscular transmission.

Curare is a generic term for various South American arrow poisons that used for killing wild animal for food. The death was resulted from the paralysis of skeletal muscle. Curare can be extracted from various South American species of *Strychnos*, for instance, *Strychnos toxifera*. Many investigators used curare as the important tool to demonstrate the unique aspects of nerve-to-muscle signaling at neuromuscular junction.

"Kloi", the plant of family Dioscoreaceae exhibited a depressive effect on neuromuscular transmission in rat phrenic nerve-hemidiaphragm like a non-depolarizing neuromuscular blocking agent, d-tubocurarine (Ridtitid, 1977).

Chaichana and Apisariyakul (1996) reported that the "quercetin" extracted from natural source, such as the leaves of *Psidium guajava* (Myrtaceae) possessed the cholinergic blocking effect. They proposed that quercetin exerts its effect on the

neuromuscular junction due to depolarized blocking action at the postsynaptic site and/or a decrease in sensitivity of motor endplate to acetylcholine.

Achola and Munenge (1997) reported that the crude extract of root and aerial parts of *Ageratum conyzoides* were found to have neuromuscular blocking activity in isolated rat phrenic nerve-hemidiaphragm. In addition, this plant extract also demonstrated the Ca^{2+} channel blocking activity.

Das and colleagues (1997) demonstrated the effect of hot water extract of the green tea (*Camellia sinensis*) on skeletal muscle and its neurotransmission using isolated rat phrenic nerve-hemidiaphragm preparation. It has a facilitatory effect at lower concentrations and a paralytic effect at higher concentrations and it was suggested that the extract might act on Ca^{2+} channels at the neuromuscular junction.

The species of interest in the present study is *Piper sarmentosum* Roxb. which is also known in local name as "Chaplu". According to the Thai traditional recipes, it was used as a carminative and an expectorant, and also used to relieve abdominal and muscle pain, cough, and to relieve the severity of traumatic injury (Pongboonrod, 1976; Li Ning-hon, 1980). In the Malay and Indonesian Archipelago, the leaves and roots of this species have been use as an

effective remedy for toothache, fungoid dermatitis on the feet, coughing, asthma and pleurisy (Perry, 1981).

It was reported that the crude water extract of *Piper sarmentosum* Roxb. could reduce blood glucose in alloxan-induced diabetic rabbits. Furthermore, administration of the extract to maturity-onset diabetic patients resulted in a reduction of blood glucose level (Pongmarutai, 1980).

Sunbhanich and colleagues (1988) studied on the effects of the crude methanol extract on isolated guinea pig ileum and isolated rat phrenic nerve-hemidiaphragm preparation. They proposed that the extract acted like a cholinergic agonist on ileum and a depolarized neuromuscular blocking agent on the hemidiphragm preparation.

According to the preliminary results of Sunbhanich and colleagues (1988), the studies are still not enough to support the profile of responses, interactions between the extract and the standard neuromuscular blocking agents, and the sites of action at neuromuscular junction. Therefore, the purposes of this investigation are extended to study the more details of effects of the crude methanol extract of *Piper sarmentosum* Roxb. in the isolated rat phrenic nerve-hemidiaphragm preparation. The experiments have been focused on two aspects of neuromuscular activity of the extract. Firstly, the experiments have been examined for dose-response relationships and the possible sites of action of the extract in both neurally- and directly-

evoked twitch. Secondly, the experiments have been studied to verify the possible interaction of the extract with standard neuromuscular blocking agents.

CHAPTER 2

REVIEW OF LITERATURES

Piper sarmentosum Roxb. (Piperaceae)

Trease and Evans (1983) described that the Piperaceae consists of 4 genera and 2,000 species. The plants are tropical, mostly climbing shrubs or lianas, with swollen nodes and fleshy spikes of flowers. The leaves contain oil cells. The one-celled ovary has a single ovule and develops into a berry. The seeds contain endosperm and abundant perisperm.

Piper sarmentosum Roxb., local name "chaplu" (Figure 1), is a terrestrial herb, 1-2 feet high, jointed at the nodes. The leaves are thin, dark green and heart-shaped, and widely distributed throughout Thailand. The plant is classified into the family of piperaceae. The leaves of *Piper sarmentosum* are used as vegetable or food wrapping. According to Thai traditional medicine, this plant is used as an expectorant, carminative and in the treatment of diabetes mellitus. The fruit was also used in the treatment of asthma (Pongboonrod, 1976).

Perry (1981) reviewed for their ethnomedical properties and reported that it was used to reduce fever and to aid in digestion. The root is prepared to a remedy for toothache and may be made into a

Figure 1. *Piper sarmentosum* Roxb. (Piperaceae)

wash for fungoid dermatitis on the feet. In Malay Peninsula, leaves are applied to the forehead of children with headache. A decoction of the boiled leaves may be utilized as an embrocation and rub to cure weakness and pain in the bones, and further, discoloration of skin. In Indonesia, the rootlets chewed with betel nut and the juice swallowed is beneficial for neglected cough and asthma; chewed with ginger to treat toothache; and chewed with little nutmeg and ginger to treat pleurisy. At the same time warmed leaves coated with coconut oil are applied to the painful chest.

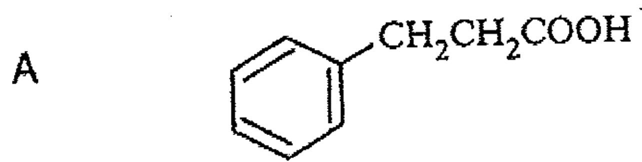
Chemical constituents

Niamsa and Chantrapromma (1983) showed that the petroleum ether leaves extraction yielded hydrocinnamic acid and *β*-sitosterol (Figure 2).

Likhitwitayawuid and colleagues (1987) reported that six components were isolated from the fruit of *Piper sarmentosum*. Two of them are the ubiquitous *β*-sitosterol and the known unsaturated amide. The other four components, which are new natural products, consist of the aromatic alkene, the pyrrole amide and two unsaturated pyrolidine amides, which were named as sarmentine and sarmentosine.

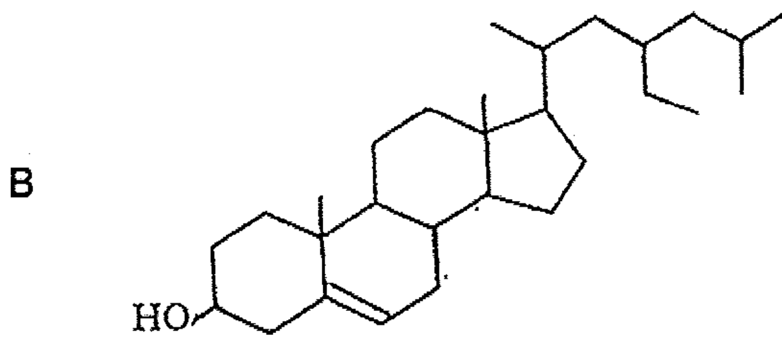
Masuda and colleagues (1991) identified the benzene soluble fraction of the methanolic leave extract to give four phenylpropanoids. They are 1-allyl-2,4,5-trimethoxybenzene, 1-(1-*E*-

A



hydrocinnamic acid

B



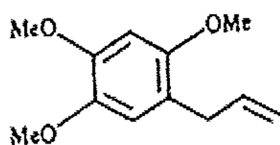
β-sitosterol

Figure 2. The molecular structure of hydrocinnamic acid (A) and *β*-sitosterol (B).

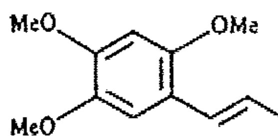
propenyl)-2,4,5-trimethoxybenzene, 1-allyl-2-methoxy-4,5-methylenedioxybenzene, and 1-allyl-2,6-dimethoxy-3,4-methylenedioxybenzene (Figure 3). The same authors revealed that the compound, 1-allyl-2,4,5-trimethoxybenzene is asarone. The compound, 1-(1-*E*-propenyl)-2,4,5-trimethoxybenzene is α-asarone, which has been isolated from the fruit of the plant. The compound, 1-allyl-2-methoxy-4,5-methylenedioxybenzene is asaricin and 1-allyl-2,6-dimethoxy-3,4-methylenedioxybenzene is a new natural product.

Strunz and Finlay (1995) synthesized sarmentosine that isolated from fruit of *Piper sarmentosum*. It was synthesized by a short efficient pathway in which the essential steps was an aldol-Grob fragmentation sequence, and the overall yields was 21% (Figure 4).

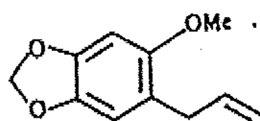
Aunpak and colleagues (1997) examined chemical composition of essential oils distilled from the leaves and fruits of *Piper sarmentosum* which was achieved by GC/MS and analyzed from the retention times compared with terpenes library revealed that longifolene (24.30 %), β-caryophyllene (10.11 %), *allo*-aromadendrene (13.51%) and *9-epi-(E)*-caryophyllene (18.24 %) were the major constituents of the leaf oil whereas β-caryophyllene (31.11 %), β-asaron (26.65 %), viriflorene (9.28 %) and β-selinene (8.21 %) were the major constituents of the fruit oil.



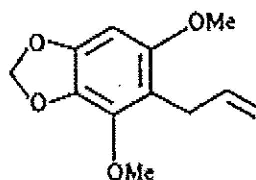
1-allyl-2,4,5-trimethoxybenzene



1-(1-*E*-propenyl)-2,4,5-trimethoxybenzene



1-allyl-2-methoxy-4,5-methylenedioxybenzene



1-allyl-2,6-dimethoxy-3,4-methylenedioxybenzene

Figure 3. The molecular structure of four phenylpropanoids.

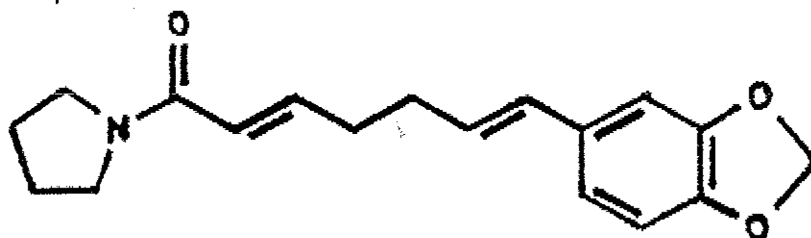


Figure 4. The molecular structure of sarmentosine.

Pharmacological activities

Pongmarutai (1980) showed that the crude water extract of *Piper sarmentosum* could reduce blood glucose only in alloxan-induced diabetic rabbits but it has no effect in normal fasted rabbit. The administration of the extract to maturity-onset diabetic patients resulted in a reduction of blood glucose level. However, the methanol extract from *Piper sarmentosum* could not reduce blood glucose in normal rabbits.

Apisariyakul and Anantasarn (1984) studied on the pharmacological activity of eleven Thai medicinal plants used as cathartics and antispasmodics in isolated rat ileum. They found that *Piper sarmentosum* decreased the intestinal tension and also inhibits the acetylcholine-induced intestinal tension.

Sunbhanich and colleagues (1988) reported the pharmacological effect of the crude methanol extract of *Piper sarmentosum* on the isolated guinea pig ileum and the isolated rat phrenic nerve hemidiaphragm preparation. The results showed that the extract could produce an increase in both frequency and amplitude of contraction of ileum while atropine could only slightly antagonize that effect. In rat phrenic nerve-hemidiaphragm preparation, the extract produced a slight twitch potentiation followed by twitch depression. They concluded that the extract acted like a

cholinergic agonist on the ileum and a depolarizing neuromuscular blocking agent on the neuromuscular junction.

Masuda and colleagues (1991) reported that the benzene soluble fraction of the methanolic leave extract, which was purified to give four phenylpropanoids, showed antimicrobial activity against *Escherichia coli* and *Bacillus subtilis*.

Aunphak and colleagues (1997) reported that essential oils, distilled from the leaves and fruits of *Piper sarmentosum*, were active against *Pseudomonas aeruginosa, Bacillus subtilis* and *Candida albican*.

The Neuromuscular Junction

The neuromuscular junction (NMJ) is a chemical synapse that is the point at a nerve ends in a muscle, at which the signal is transmitted from the motor nerve terminal to an adjacent postsynaptic region on the muscle fiber. It's formed by 5 principle components as followings (Engel, 1994).

1. A Schwann cell process that forms a cap above that portion of the nerve terminal which does not face the postsynaptic region.
2. A nerve terminal which contains neurotransmitter.
3. A synaptic space which lined with basement membrane.
4. A postsynaptic membrane which contains the receptors for the neurotransmitters.
5. The junctional sarcoplasm which provides structural and metabolic support for the postsynaptic region (Figure 5).

They can be divided into three regions, i.e., (1) presynaptic region, (2) synaptic space, and (3) postsynaptic region.

In vertebrate voluntary muscle, the neurotransmitter is acetylcholine (ACh), the receptor is acetylcholine receptor (AChR), and the synaptic space contains acetylcholinesterase (AChE).

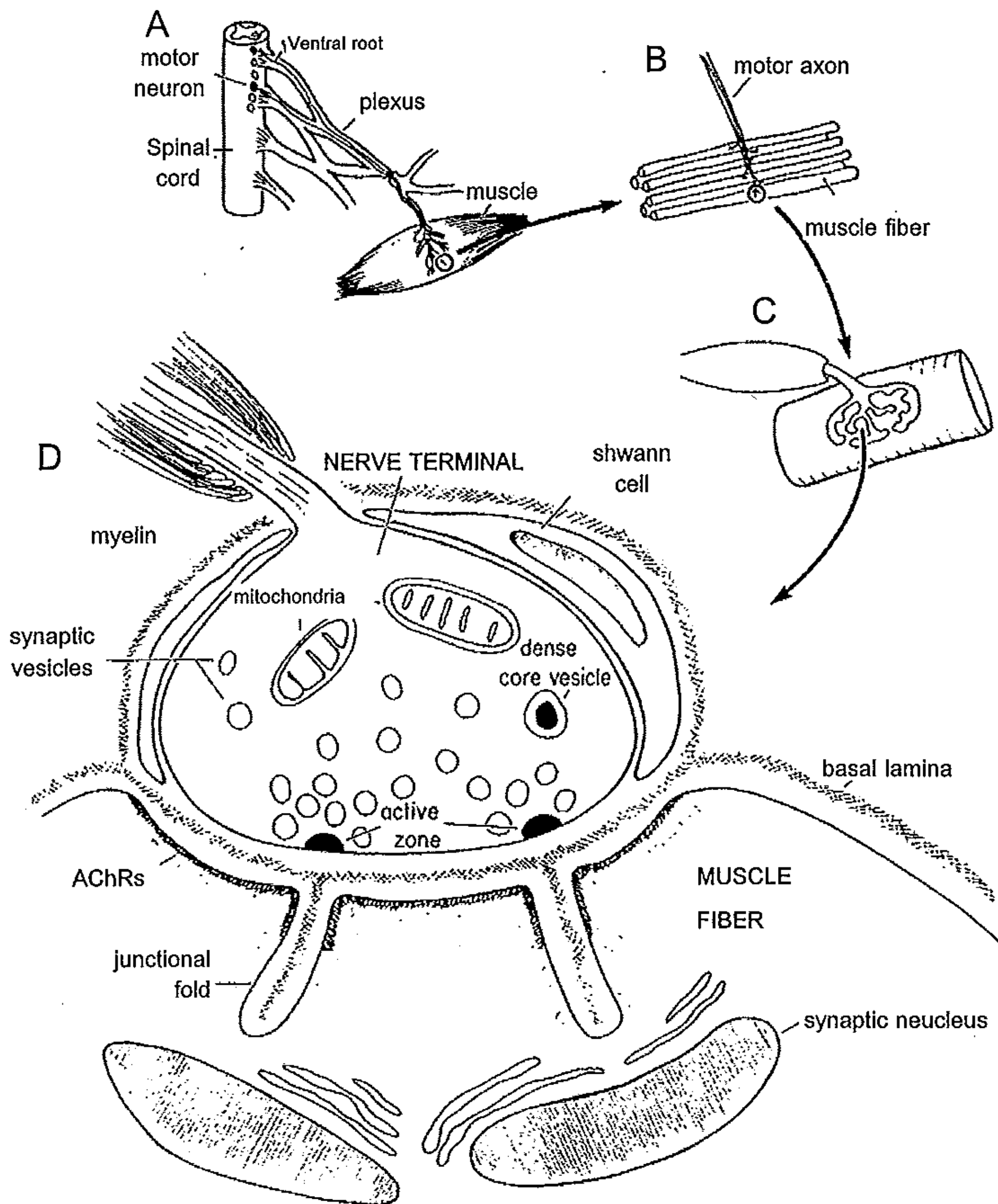


Figure 5. The neuromuscular Junction.

Source: Hall and Sane, 1993: 101.

Skeletal muscles are innervated by motor neuron, whose cell bodies lie in the spinal cord or brain stem. Each of them sends an axon through a plexus of peripheral nerve to a single muscle (Figure 5A). The motor neuron axons are myelinated and have the largest diameter axons in the body. Therefore, they are able to propagate action potentials at high velocities, allowing signals from the central nervous system to be transmitted to skeletal muscle fibers with minimum delay.

As each axon of a motor neuron arrives at its target fiber, it loses its myelinated sheath and makes a spray of fine terminal branches (Figure 5B). Each branch forms a single junction with a muscle fiber (Figure 5C). Thus, a single motor neuron innervates many muscle fibers but each muscle fiber is controlled by only one motor neuron. A motor neuron plus the muscle fibers its innervates is called a *motor unit*. The nerve terminals lie in shallow gutter formed by the sarcolemma of muscle fibers and are capped by processes of Schwann cells (Figure 5D). In mammals, most muscle fibers are innervated at a single synaptic site by a single axon. As a consequence, no synaptic integration occurs at the neuromuscular junction; each action potential in the nerve terminal causes a single action potential in the muscle fiber (Hall and Sanes, 1993).

The Presynaptic Region

The preterminal myelinated nerve fiber is surrounded by a sheath of perineural epithelial cell (Henle' s sheath) which is partially surrounded by fibroblasts and other connective tissue elements. The myelinated sheath ends abruptly at the last node of Ranvier. Between the last node of Ranvier and the NMJ, the terminal axon is enveloped by the Schwann cell which surrounded by Henle' s sheath. Henle' s sheath ends a short distance above the NMJ but the Schwann cell extends to cover that aspect of the nerve terminal which does not face to the postsynaptic region (Hall and Sanes, 1993; Engel, 1994).

Hall and Sanes (1993) described this covering that it presumably protects the nerve terminal from chemical and mechanical insults. However, three sets of observation suggest that terminal Schwann cell may play other roles as well. First, they elaborate extensive processes and phagocytize nerve terminal following axonal damage and thus may participate in axonal remodeling. Second, they exhibit a calcium transient in response to axonal action potentials, showing that they can sense electrical signals. Finally, they acquire the ability to synthesize and secrete acetylcholine following denervation and are thus capable to transmitting signal to muscle.

The nerve terminal contains not only mitochondria and other common subcellular structures, but also the site of accumulation of

about 50 nm diameter synaptic vesicles which contain the neurotransmitter, ACh. The vesicles are more abundant near the synaptic membrane which faces the postsynaptic region than elsewhere in the nerve terminal, whereas mitochondria and other organelles are more abundant in the center and upper part of the terminal. The synaptic vesicles tend to form small clusters adjacent to dense patches on the presynaptic membrane. These patches are called *active zone* that are the site at which synaptic vesicles fuse with the presynaptic membrane (exocytosis) to release their stores of acetylcholine into the synaptic space.

The function and activities of the synaptic vesicles include (1) the concentrative uptake and storage of acetylcholine, (2) movement to and densing at the active zones, (3) fusion with the presynaptic membrane to release acetylcholine into the synaptic space, and (4) retrieval form the presynaptic membrane follow by (5) recycling (Engel, 1994).

The Synaptic Space

The 20-50 nm wide synaptic space is formed by a basal lamina which traverses between pre-and postsynaptic membrane and extends to the junctional fold (Hall and Sanes, 1993). The space is somewhat arbitrarily divided by Engel (1994) into a primary and a

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number of secondary clefts. The primary clefts are limited by the presynaptic membrane on one side and, on the opposite side. It lacks lateral boundaries except basement membrane and, therefore, communicates with the extracellular space. The secondary clefts are space between the junction fold, and each secondary cleft communicates with primary clefts.

Although the basal lamina is morphologically indistinguishable from the extrasynaptic basal lamina to which it is attached, it is biochemically specialized and it contains acetylcholinesterase (AChE) which inactivates the transmitter, as well as components responsible for the strong adhesion of nerve to muscle and factors that mediate developmental interactions (Hall and Sanes, 1993).

The Cholinesterase Enzymes

The acetylcholine, once released into the synaptic space, continues to activate the acetylcholine receptors as long as it persists in the synaptic space. However, it is rapidly removed by two ways (Guyton, 1991):

1. Most of the acetylcholine is destroyed by the enzyme acetylcholinesterase (AChE) that is attached mainly to the basal lamina, a spongy layer of fine connective tissue that fills the synaptic space between pre-and postsynaptic membrane. Hence, AChE, which

catalyzes the inactivation of acetylcholine with the production of choline and acetate.

2. A small amount diffuses out of the synaptic space and is then no longer available to act on the muscle fiber membrane.

Lefkowitz, *et al.*, (1991) described that cholinesterase was two types found, there are (1) acetylcholinesterase (AChE: also known as specific or true ChE) is found in cholinergic neurons and highly concentrated at the neuromuscular junction. (2) Butyrylcholinesterase (BuChE: also known as pseudo-ChE) is present not only in neuronal element but also in the plasma, liver and other organs.

Hennis (1990) described that AChE has two active sites. The anionic site relates an overall negative charge and binds the positively charged end of the ACh molecule, orienting it properly for hydrolysis (Figure 6). The esteratic sites is responsible for the actual cleavage of the molecule. This site contains electronegative group that contributes a pair of electrons to form a bond with the acetate moiety of ACh. This ends the action of ACh and produces free choline plus a transient intermediate, the acetylated enzyme. The acetylated enzyme adds water to regenerate free enzyme and acetic acid.

Bowman (1994) described that each enzyme site is capable of hydrolyzing about 5×10^5 molecules of acetylcholine per minute. Once it has arrived at the enzyme, each acetylcholine molecule is splited in 80-100 μs; such widespread and rapid enzyme activity

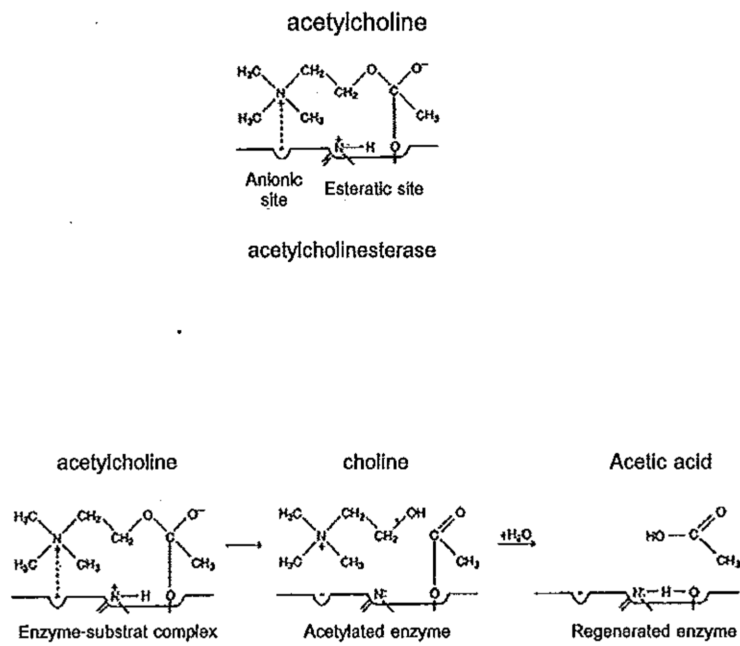


Figure 6. Hydrolysis of acetylcholine by acetylcholinesterase.

allows each acetylcholine molecule to react no more than once with an acetylcholine receptor. And ensures that there is no accumulation of released acetylcholine from one nerve impulse to the next, even during the highest possible frequencies of the nerve impulse traffic.

The Postsynaptic Region

The postsynaptic region consists of junctional fold and junctional sarcoplasm (Engel, 1994). Junctional folds are not found at other synapse except the NMJ. At synapses that lack junctional folds, the surface areas of the pre-and postsynaptic membrane are essentially identical. About 1 μM deep junctional folds produce a several fold amplification of the postsynaptic surface. Because the junctional folds are separated by secondary synaptic space.

The mouth of junctional folds are precisely aligned with the presynaptic active zone. The synaptic neuclei are morphologically different from their nonsynaptic counterparts.

The postsynaptic membrane lining the junction folds contains AChRs, voltage-sensitive sodium channels, and other membrane proteins (Hall and Sanes,1993). AChR is concentrated at the crest of the junctional folds, whereas sodium channels are concentrated in the trough of the folds and in the perijunctional region (Flucher and Daniels,1989).

The Acetylcholine Receptor

There are two types of receptor in the ligand-gated ion channel gene superfamily which exhibit nicotinic pharmacological properties for binding acetylcholine. These are muscle AChRs and neuronal AChRs. Both are the pentamer of five glycoprotein subunits (Lindstrom, 1994).

Muscle nicotinic AChRs

These AChRs are found in muscle and electric organ. They are ACh-gated cation channels which function as the critical postsynaptic link in neuromuscular transmission. They are found in two subtypes :(1) extrajunctional AChRs, which are distributed throughout the surface membrane of immature or denervated muscle, and (2) junctional AChRs, which are located only in the postsynaptic membrane at mature synapses. These two subtypes differ not only in developmental regulation and location but also in subunits composition, pharmacology, channel properties, and turnover rate.

Standaert (1991) described that junctional AChRs are confined to the endplate region of the muscle membrane, but the extrajunctional ones are not. They tend to be concentrated in the area of neuromuscular junction, but they may be inserted anywhere in the muscle membrane (Figure 7A).

Lindstrom (1994) described that muscle AChRs are composed of five homologous subunits. Each AChRs monomer

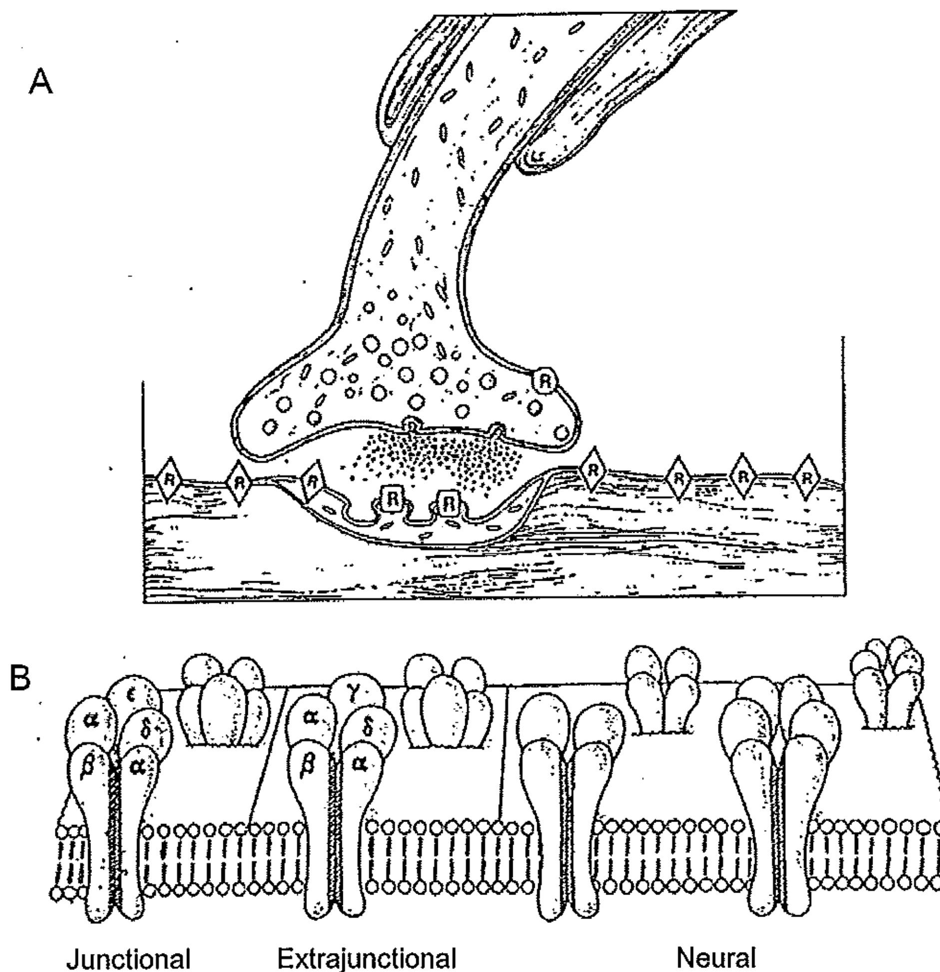


Figure 7. Sketch of the neuromuscular junction and various types of nicotinic AChRs.

A. Muscle membrane contains extrajunctional receptors (R). Endplate membrane contains both junction (R) and extrajunctional receptors. The nerve ending contains neuronal nicotinic AChRs (R).

B. The structures of junctional, extrajunctional, and neuronal nicotinic AChRs.

Source: Standeart F.G., 1991: 676.

consists of two α subunits and are identical, one β subunit, one δ subunit and either a γ subunit in extrajunctional AChRs or an ε (epsilon) subunit in junctional AChRs (Figure 7B).

Replacement of the γ subunit of extrajunctional AChRs with ε subunit of junctional AChRs is responsible for a slight increase in the conductance of the open channel and for a large decrease in the duration of bursts of channel openings caused by ACh (Lindstrom, 1994).

Furthermore, Glan and Pollard (1995) described that the ε subunit was found in adult mammalian. In the fetus and various other species, the ε subunit did not exist, it was replaced by γ subunit. However, these subunits appear to be organized like barrel stares around a central ion channel of which they are all component. The acetylcholine binding site are formed by two α subunits in combination with γ , δ or ε subunits (Lindstrom, 1994; Glan and Pollard, 1995).

The largest and probably one of the most physiologically significant differences between junctional and extrajunctional AChRs is in the rate of turnover, a half-life of 12 to 21 hours for extrajunctional AChRs, but greater than 10 days for junctional AChRs (Lindstrom, 1994).

Brenner and Sakmsnn (1983) suggested that the clinical significance of these receptors become evident when there is a

proliferation of extrajunctional receptors secondary to neural damage, muscle injury, or burn trauma. Therefore, upper motor neuron disease (stroke, spinal cord injury), lower motor neuron disease, muscular disease (some muscular dystrophies, disuse atrophy, even cast immobilization), or muscular injury and burn trauma will cause an increase in extrajunctional receptor formation. These receptors are sensitive as junctional receptors to agonist stimulation. However, their channels remain open approximately four times longer than junctional receptors.

Neuronal Nicotinic AchRs

They are ACh-gated cation channels, like muscle AChRs. They exhibit functional patterns of activation and desensitization, channel duration, and conductance in general not greatly different from those of muscle AChRs. In ganglia, they perform a postsynaptic role in transmission, like muscle AChRs, but many of the AChRs in central nervous system may be located presynaptically, where they may modulate the release of ACh or other transmitters (Lindstrom, 1994).

Standaert (1991) described that the α-bungarotoxin (a polypeptide derived from snake venom that causes neuromuscular blockade) binds to prejunctional membranes, much as it does to receptors in the postjunctional membrane. However, later studies

showed that α-bungarotoxin can be washed away from receptors in the nerve ending, whereas it binds irreversibly to endplate receptors.

This difference in reversibility of binding suggests that the receptors in the nerve ending are more like those in the central nervous system, ganglia, and other parts of the nerve system than like those in muscle. The prejunctional receptors in the nervous system seem to be cylindrical assemblages of protein subunits that span the cell membrane and, when activated, allow the passage of ions. Further, the subunits seem to be close relatives of the α- and β-subunits of the muscle receptors. However, neuronal receptors seem to be composed of only two kinds of subunits and do not include the several others found in muscle receptors. Moreover, neuronal receptors seem to be composed of an even number of subunits, for example, four or six, instead of the five that compose postjunctional receptors (Figure 7B). Several variants of each the α- and β-subunits have been found and several combinations of the variants are known, so that greater variety of receptors and pharmacologic specificities may exist in the nervous system than in muscle.

Neuromuscular Transmission

Synaptic Transmission

All neuronal type-regardless of their shape, size, location and function exhibit four anatomically distinct compartments, each of which has a distinctive role in signaling : the cell body, dendrites, an axon and the nerve terminal. The dendrite and cell body make up the input compartment of the neuron. The axon is the long-range signaling compartment.

The dendrites and cell body contain receptors and ion channel proteins that transform incoming signals into excitatory or inhibitory synaptic potentials. Excitatory synaptic potentials reduce the membrane potential, called depolarization and, if sufficiently large, bring the membrane potential to the threshold for producing an action potential, a brief all-or-none electrical signal that is about 1 ms in duration and 100-110 mV in amplitude. Inhibitory synaptic potentials increase the membrane potential, as a consequence, it acts to prevent the axon from reaching the threshold for generating an action potential that refer to hyperpolarization (Jessell and Kandel, 1993). Spatial and temporal summation of excitatory and inhibitory inputs determine whether or not the neuron fires an action potential. Once initiated, the action potential propagates without failure along the axon to its output element, the presynaptic terminals, arrival of the action potential at the

synaptic terminal leads to the release of packets (quanta) of chemical transmitter from the terminals. The transmitter diffuses across the synaptic cleft which separates the presynaptic terminal and postsynaptic target of the synapse, and interacts with receptors on the the postsynaptic cell. The properties of the individual synapse, in particular, the properties of postsynaptic receptors, determine whether the binding of transmitter leads to either an inhibitory or an excitatory synaptic potential.

According to the ionic hypothesis (Hodgkin, Huxley and Katz; reviewed by Jessell and Kandel, 1993), it can explain how the electrical potential difference across the neuronal membrane (the resting potential) is generated, and how its sign is quickly reduced and then reversed during the generation of action potential. The signaling capabilities of nerve cell derive from two families of specialized membrane proteins-channels and pumps that permit ions to cross the membrane. Pumps actively transport specific ions against an electrochemical gradient and therefore require metabolic energy. Channels are water-filled protein pores in the lipid bilayer that permit specific ions to move rapidly down their electrochemical gradient and do not require metabolic energy.

Channel proteins fall into two classes. Voltage-gated channels sense the electrical field across the membrane and open in response to changes in this potential. Some of these channels are only

modestly voltage dependent and are open at the resting level of the membrane potential. These channels, called leakage channels, contribute to the resting potential. Other channels are not open at rest and are markedly voltage dependent. These contribute to the action potential, ligand-gated channels recognize chemical transmitters and open when a specific transmitter is bound to them. These produce synaptic potentials.

Nerve cells at rest maintain a potential difference across their surface membrane, with cytoplasmic face more negative than the external face by about -70 mV. The ionic hypothesis proposed that this potential difference results from an unequal distribution of Na^+, K^+, Cl^-, and other organic anions (mostly proteins) across the membrane combined with a set of leakage K^+ channels that are open at resting membrane potential. There is a high concentration of K^+ inside the nerve cell and a low concentration outside. The asymmetric distribution of ions across the membrane is maintained by a Na^+-K^+ pump (Na^+-K^+ ATPase) that transport Na^+ out of and K^+ into the cell ($3Na^+$ for $2K^+$), keeping the K^+ concentration within the cell about 20 times higher and the Na^+ concentration about 10 times lower than those outside the cell. Because it is concentrated in the cell, K^+ tends to diffuse out of the cell, through the leakage K^+ channel, under the influence of its concentration gradient, thereby making the inside surface of the cell membrane slightly more negative than outside. The

buildup of negative charge on the inside surface of the membrane impedes the further movement of positively charged K^+ ions. At a certain potential, the force provided by the electrical charge that built upon the membrane is equal to that of the oppositely directed force that results from the K^+ concentration gradient, and, as a results, no further net movement of K^+ occurs. The potential at which this occurs is called the resting membrane potential and is usually about -70 mV (Jessell and Kandel, 1993).

Most cells in the body generate at a resting potential across their surface membrane. The resting membrane potential can be altered to serve as a signaling mechanism. When the membrane potential of a nerve cell is reduced by a critical amount, usually about 15 mV (from -70 mV to -55 mV), a regenerate, self-limiting, all-or-non action potential of 100-110 mV amplitude is initiated (Jessell and Kandel,1993) and repolarized reset itself to resting stage (Figure 8).

The action potential is generated as a result of the sequential activation of two classes of voltage-gated channel, one for Na^+ and the other for K^+. At rest , both classes of channels are closed , but both are opened with depolarization : the Na^+ channels is opened rapidly, and the K^+ channel is opened with delay. The opening of Na^+ channels admits Na^+ into the cell, which, in turn, reduces the negative charge on the inside surface of the membrane and causes further depolarization. The resulting potential change is regenerative,

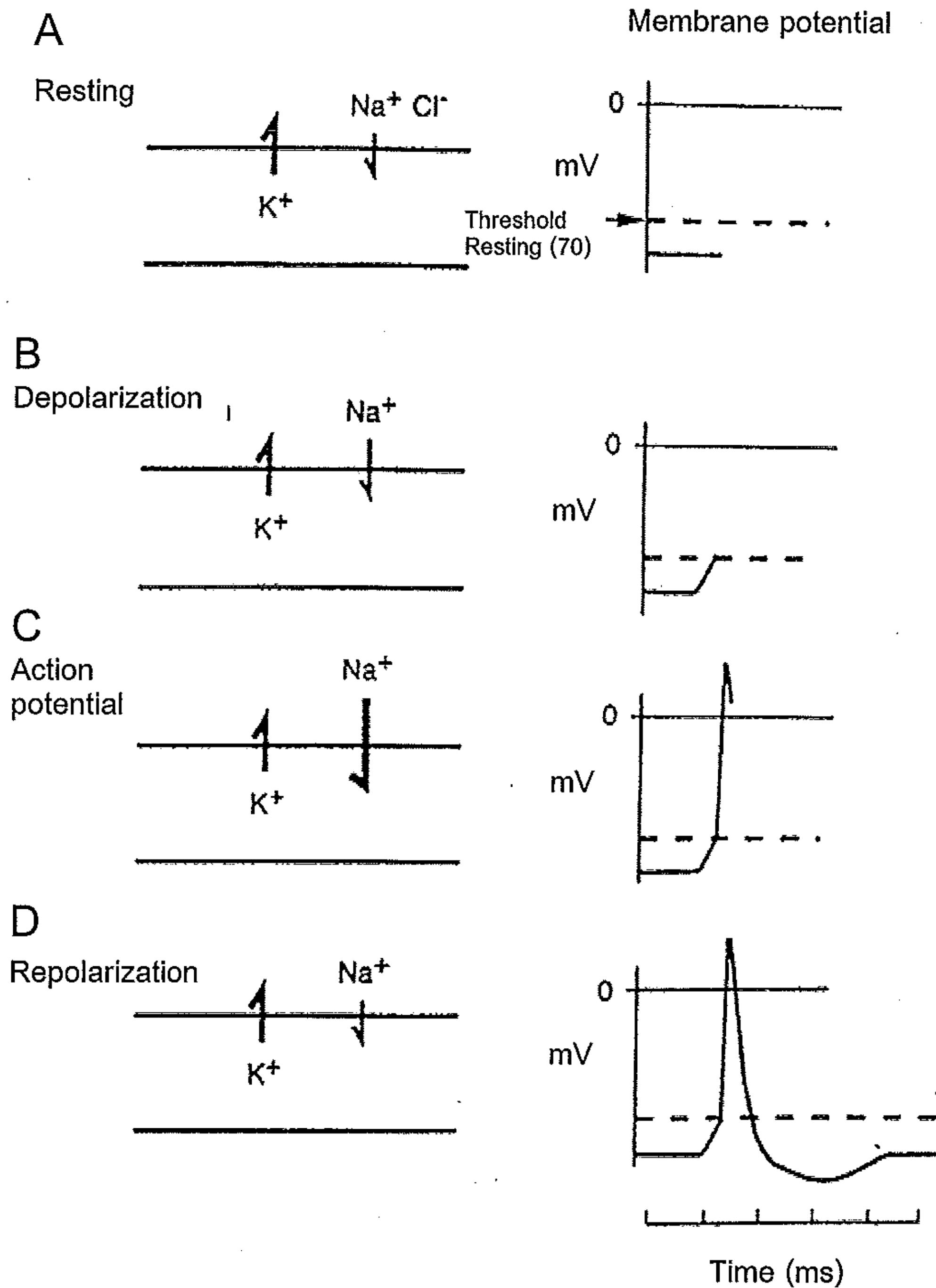


Figure 8. The events changing ionic permeability on membrane potential.

in that further depolarization open additional Na^+ channels. This explosive event abolishes and reverse the resting potential, ultimately driving the membrane potential toward the Na^+ equilibrium potential. The sudden reversal of the membrane potential is transient and self-limiting. The combined effect of two processes terminates the action potential and brings the membrane back to the resting level. First, the progressive depolarization of the action potential and the consequent opening of the Na^+ channels also lead to the inactivation of the voltage-dependent Na^+ channels. Second, after a short delay, the depolarization opens voltage-gated K^+ channels.

Once initiated, the action potential generates a local current flow that is sufficient to depolarize the adjacent region of the membrane, in turn triggering an action potential. When the action potential is propagated without decrement along through the axon, the depolarization in the nerve terminal is produced. (Jessell and Kandel, 1993)

Synthesis and Storage of Acetylcholine

Acetylcholine was synthesized within the nerve terminal (Figure 9) from reaction of acetyl coenzyme A and choline and under the influence of the catalyzing enzyme choline-0-acetyltransferase

(Miyamoto, 1978). Much of the acetate is synthesized in the mitochondria and it combines with acetyl coenzyme A, which is

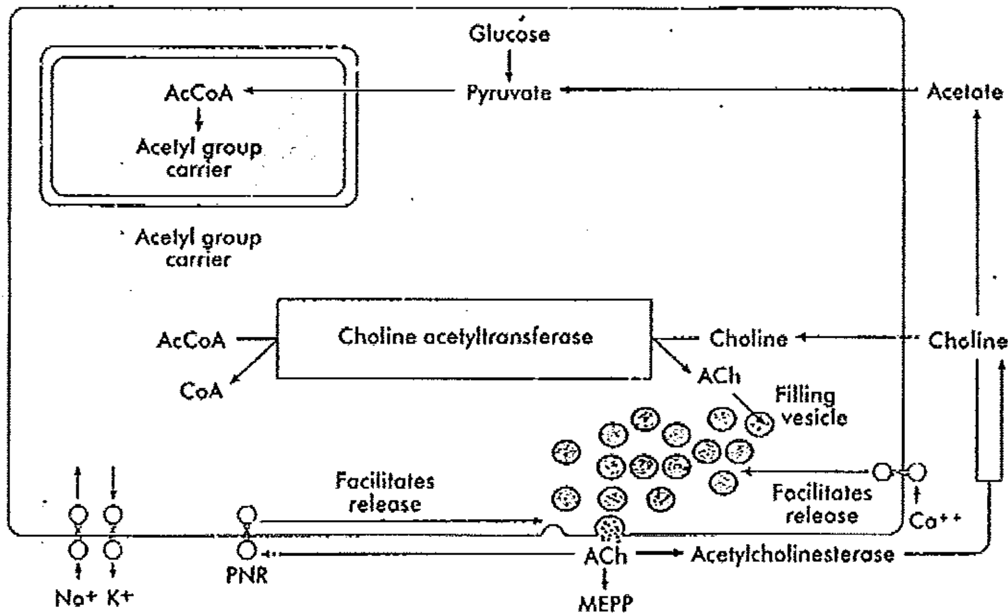


Figure 9. The diagram of synthesis, storage and release of acetylcholine. AcCoA, acetylcoenzyme A; CoA, coenzyme A; ACh, acetylcholine; PNR, perijunction nicotinic receptor, MEPP, miniature endplate potential.

Source: Haspel K.L, et al., 1993: 1510.

produced by the neuron (as well as by most cells) in the axoplasm. However, acetate derived from the hydrolysis of the released transmitter is also utilized. It is taken up into the nerve endings by a specific transport process. Choline is derived from the extracellular fluid and enters the axoplasm by means of a high-affinity sodium ion-dependent transport mechanism that is present in the membranes of all cholinergic nerve terminals (McIntosh and Collier, 1976). The plasma membrane of the motor neuron has a transport system, powered by the Na^+ gradient, that can accumulate choline against a large electrochemical potential gradient. About half the choline that is freed in the synaptic cleft when acetylcholine is hydrolyzed is actively taken back up into the motor neuron to be used in the resynthesis of acetylcholine (Berne and Levy, 1993).

After synthesis; it is stored in synaptic vesicles containing a quantum (several thousand molecules of ACh) (Birks, *et al.*, 1960; Foldes, *et al.*, 1989). A part of these vesicle are assumed to be in close proximity to the prejunctional membrane of the motor nerve terminal, and are readily available for release by the nerve impulse (Elmquist and Quastel, 1965 ; Foldes, *et al.*, 1989). As the readily available store of ACh is used up it is replenished from a larger reserve store located at some distance from the prejunctional membrane (Bowman, 1980; Foldes, *et al.*, 1989).

Release of actylcholine

The depolarization of the terminal membrane during the action potential leads to the release of ACh by the entry of Ca^{2+} ions from the external medium through voltage-gated Ca^{2+}-channels which opened by membrane depolarization (Lingle and Steinbach, 1988). The Ca^{2+}channels close when the membrane repolarizes after the action potential. The initial depolarization of the terminal results from the opening the voltage-gated Na^{+} channels in the terminal membrane (Konishi, 1985), and the resulting entry of positive ions. The repolarization that terminates the action potential (and hence limits the period of Ca^{2+} entry) results in part from the inactivation of Na^{+} channels, but largely from the opening of voltage-gated Ca^{2+} activated K^{+} channels (Mallart, 1985 ; Braga, *et al.*,1994). Therefore, the amount of Ca^{2+} entering during an action potential and the duration of the entry depend not only on the opening of Ca^{2+} channels, but also the Na^{+} and K^{+} channels (Lingle and Steinbach, 1988). The resultant influx of Ca^{2+} produce localized accumulation of Ca^{2+} in the presynaptic terminal near the release site. This local increase in Ca^{2+} concentration greatly enhance the probability of vesicle fusion and neurotransmitter, acetylcholine, release by exocytosis (Jessell and Kandel, 1993).

Lingle and Steinbach (1988) had described that the mechanism of ACh release is thought to involve the fusion of small vesicles in the nerve terminal with the surface membrane of the

terminal, resulting in the release of ACh contained in the vesicles by exocytosis. The total release of ACh is the sum of number of quanta, where each quantum is likely to correspond to a single vesicle fusion. Approximately 100 quanta are released per terminal action potential, far fewer than the total vesicle population of the nerve terminal (100,000 to 1 million). Each quantum is composed of about 10,000 ACh molecules. The number of quanta release per presynaptic action potential depends on the history of stimulation, and can show decreases (depression) and several type of increase (facilitation, augmentation, potentiation) during trains of stimuli.

Bowman (1980) had suggested that the mobilization of ACh stored in synaptic vesicles be facilitated by positive feedback mechanism through nicotinic receptor located on the motor nerve terminal. Nerve ending nicotinic receptors may function in a positive feedback mechanism that mobilizes transmitter fast enough to keep step with the demands of high frequencies (>1 Hz) or nerve impulses. Mobilization includes all those processes that lead to refilling of the store of transmitter that is available for release by nerve impulse, and to the renewed availability of release sites. Mobilization is therefore an important factor in release of transmitter when the frequency of nerve impulse is high. Stimulation of the prejunctional acetylcholine receptors is not regarded as directly evoking release. Such a process would lead to an unstable and unbreaked explosive vicious circle of

transmitter output. Functional release still occurs only in response to a nerve impulse, but by facilitating the mobilization process, the released transmitter balance elegantly the availability against the demand.

Activation of the ACh receptor occur as follows : a molecule of ACh binds to each of the two ACh-binding site on two single ACh receptor. When two agonist molecule are bound, the protein can then undergo a pore allowing cations to pass through the membrane (Na^{+} and Ca^{+} inward, K^{+} outward) result in a transient depolarization of the endplate region. The transient depolarization is called *endplate potential (EPP)*. The EPP is transient because the action of ACh is terminated by acetylcholinesterase (AChE), which is present in high concentration on the postjunctional membrane. The postjunctional plasma membrane of the neuromuscular junction is not electrically excitable and dose not fire action potential. After it is depolarized, adjacent regions of the muscle cell membrane are depolarized by electrical conduction. When those regions reach threshold, action potential are generated. Action potential are propagated along the muscle fiber at high velocity and induce the muscle cell to contract.

In absence of nerve impulses, a spontaneous release of acetylcholine occurs in packages or quanta of uniform size. The quanta give rise to small and transient depolarizations of the postjunctional membrane. These are the *miniature endplate potentials*

(m.e.p.p.). Their function is unknown, but they represent the spontaneous and random occurrences of single units of the much larger multiple response that is triggered by the nerve impulse. A m.e.p.p. is much too small to excite the muscle fiber membrane and therefore does not give rise to contraction. The frequency of occurrence of m.e.p.p.s is increase when the nerve endings are depolarized, and their occurrence is dependent upon the presence of calcium ions. However, they are less dependent upon extracelluar calcium ion concentration than is the nerve impulse-evoked release. This is because the axoplasm already contains enough calcium ions to support much of the spontaneous quantal release.

The general mechanism of neuromuscular transmission may be summarized as follows (Figure 10):

1. An action potential propagating down the motor axon invades and depolarizes the presynaptic nerve terminal.

2. Voltage-dependent calcium channels in the presynaptic nerve terminal open in response to the depolarization.

3. Ca^{2+} move down its electrochemical gradient into the nerve terminal from the extracellular solution.

4. The influx of Ca^{2+} leads to a transient increase in the probability of transmitter release by increasing the probability that the synaptic vesicle in the nerve terminal will fuse with the presynaptic

membrane and releasing their quantal packets of acetylcholine into the synaptic cleft.

5. The released ACh diffuses toward the postsynaptic membrane and the edges of the synaptic cleft. In the process it binds with ACh receptors on the muscle membrane and is also hydrolyzed by acetylcholinesterase (AChE).

6. The AChRs that bound ACh undergo a conformational change and open their channels.

7. The channels, which have an effective open time of about 1 ms, are permeable to Na^+, K^+, and, to a lessor extent, Ca^{2+}. Move Na^+ moves through the open channels into the muscle fiber than K^+ move out, which leads to a net influx of positive charge that depolarizes the fiber, producing the end plate potential (EPP). The net current that flows through the open channel is call the end plate current (EPC).

8. When the EPP depolarizes the membrane of the muscle fiber to threshold, an action potential is generated in the muscle membrane that propagates in both directions in the muscle fiber away from the end plate, leading to contraction.

9. The Ca^{2+} from extracellular and choline from the hydrolyzed ACh is taken up by the nerve terminal for resynthesis into ACh and filled synaptic vesicle containing the quantal packets of ACh

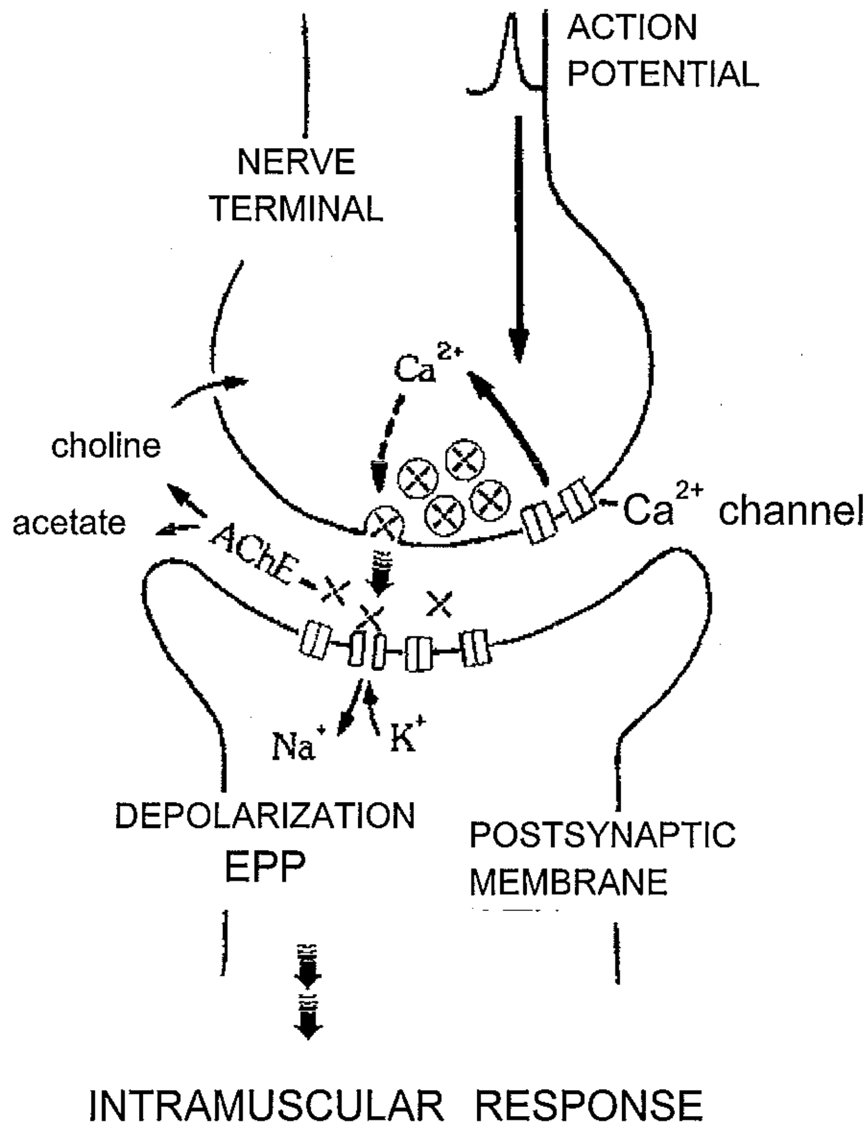


Figure 10. Summary of events occurring during neuromuscular transmission. X, acetylcholine; AChE, acetylcholinesterase; EPP, endplate potential.

are positioned at the release sites, and the membrane of the empty synaptic vesicle is recycled.

The Margin of Safety of Neuromuscular Transmission

The integrity of neuromuscular transmission is dependent on the interaction of ACh, release by the nerve impulse, with sufficient number of cholinergic receptors of the postjunctional membrane. The neuromuscular junction have excess receptor and the nerve also release more quantal packets of ACh (about 5 to 10 times) by the nerve impulse than that necessary for the depolarization of the postjunctional membrane, and the development of the endplate-and action-potential, when all the cholinoceptors are free to interact with ACh. Furthermore, if the release of ACh is not inhibited, as long as at least 30 % of the postjunctional cholinergic receptors are available for interaction with ACh, neuromuscular transmission will be unimpeded at slow stimulation rate (Paton and Waud, 1967; Foldes, *et al.*, 1989). That is the excess receptor and excess ACh give rise to a safety factor or high margin of safety of neuromuscular transmission. Foldes and colleagues (1989) had suggested that, at slow stimulation rates, the nicotinic feedback mechanism of ACh mobilization further increase the margin of safety of neuromuscular transmission.

Pharmacology of Neuromuscular Transmission

It is possible to modify the neuromuscular transmission in a number of ways. Substances that do affect neuromuscular transmission may act any one or more of a number of sites as follows (Aglan and pollard, 1995):

1. Prevention of the arrival of the nerve action potential (*e.g.,* local anesthetics).

2. Inhibition of acetylcholine mobilization and release (*e.g.,* *Botulinum* toxin, aminoglycoside antibiotics).

3. Inhibition of the uptake of choline (*e.g.,* hemicholinium).

4. Inhibition of the synthesis of acetylcholine (*e.g.,* triethylcholine)

5. Inhibition of the acetylcholine loading into the vesicles (*e.g.,* vesamicol).

6. Inhibition of the combination of acetylcholine with the postjunctional receptors (*e.g.,* d-tubocurarine).

7. Activation or inhibition of prejunctional receptors (*e.g.,* d-tubocurarine).

8. Inhibition of AChE, thereby increasing the amount of acetylcholine (*e.g.,* neostigmine).

However, neuromuscular blocking agents refer usually to the interruption of transmission between nerve and muscle that is

produced by agents that combine in a highly selective way with the recognition sites of acetylcholine receptors. The neuromuscular blocking agents may be divided into 2 groups.

1. The depolarizing (non-competitive) neuromuscular blocking agents.

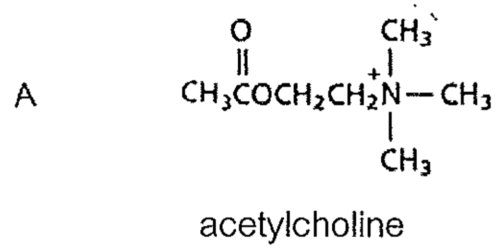
2. The non-depolarizing (competitive) neuromuscular blocking agents.

The depolarizing neuromuscular blocking agents

Agents of this type are agonists or cholinoceptive site activators, including nicotine and acetylcholine itself, are capable of blocking neuromuscular transmission. The type of block is known as block by depolarization and was first described in detail by Paton and Zaimis during their study of a series of polymethylene bistrimethylammonium compounds [$Me_3{}^+N\text{-}(CH_2)_n\text{-}N^+Me_3$; the methonium compounds] of various depolarizing blocking drugs that eventually become available, succinylcholine (suxamethonium) is the only one to persist in widespread use (Bowman, 1994).

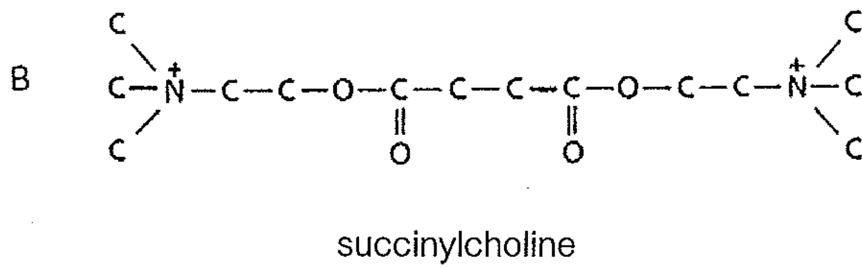
Succinylcholine is bisquaternary ammonium compound, which has a similar chemical structure to two molecules of acetylcholine linked together via the acetyl moieties (Figure 11).

A



acetylcholine

B



succinylcholine

Figure 11. The chemical structure of acetylcholine (A) and succinylcholine (B).

Standaert (1991) described that if two molecules of agonist (acetylcholine and/or a depolarizing blocking agent) attach to the acetylcholine recognition sites of the receptor. The channel will open and pass current that causes the endplate to depolarize. The agonists attach only briefly to the receptor, so each opening of a single channel is very short, about 1 ms or less. There is little difference between acetylcholine and depolarizing blocking agent either in effect or in duration of effect. Acetylcholine is rapidly destroyed by acetylcholinesterase in the junction. Thus, the cleft is clear of the transmitter and reset to a resting state before another nerve impulse arrives. In contrast, neither succinylcholine nor decamethonium is susceptible to hydrolysis by acetylcholinesterase. The drugs are not eliminated from the junctional cleft until they are eliminated from the plasma. Thus, they react repeatedly with receptors and repeatedly open channels and continuously depolarize the endplate. This comes about because of the juxtaposition at the edge of endplate of two different kinds of membrane, that of the endplate and that of the muscle, each of which has a different kind of ion channel. The membrane of the endplate contains receptor/channels that open when they bind acetylcholine or a similar chemical. The membrane of the muscle contains sodium channels that do not response to chemicals but open when they are exposed to transmembrane voltage change.

Thus, the channels in the two parts of the membrane respond to different stimuli, chemical and electrical.

The sodium channel, like the acetylcholine receptor/channel, is a cylindrical protein that can form a tube across the membrane for sodium ions to flow through. However, unlike the acetylcholine receptor/channel, it is not responsive to chemical. Only a sharply changing electrical field can cause it to open its tube. Moreover, two parts of its structure act as gates that allow or stop the flow sodium ions (Katz and Messinso, 1982).

Standaert (1991) further described that because of acetylcholine is hydrolyzed quickly, and the depolarization of the endplate is brief. The flow of ions through the receptor/channels of the endplate causes the endplate to depolarize, and the electrical effects of the depolarization extend to and influence the sodium channels in the adjacent perijunctional membrane. These open and depolarize the membrane. The depolarization spreads from one sodium channel to the next so that a wave of depolarization spreads along the muscle and triggers muscle contraction. Upon the hydrolysis of acetylcholine, ions flow across the endplate stop, the endplate membrane repolarizes, and the systems reset themselves, return to their resting stage. In contrast, when the systems expose to a depolarizing blocking agent, the initial response is like acetylcholine, but since the agent is not hydrolyzed, depolarization of the endplate is not brief.

Shortly after the voltage gate in adjacent sodium channels is opened, the time-dependent inactivation gate will close. Whereas the endplate continues depolarized, causes to the voltage gate of sodium channels still open and time-dependent inactivation gate stay closed. Hence, sodium ions cannot flow through these channels, this segment of perijunctional membrane dose not depolarize. In effect, the channels down stream are freed of depolarizing influence, the perijunctional zone become a buffer that shields the rest of the muscle from event at the endplate. The result is that the membrane is separated into 3 zone:

1. The endplate, which is depolarized by agents.

2. The perijunctional muscle membrane in which the sodium channels are frozen in an inactivated state.

3. The rest of membrane (in which the sodium channels are in the resting state).

Since a burst of acetylcholine from the nerve cannot overcome the inactivated sodium channels in perijunctional zone, neuromuscular transmission is blocked.

Bowman (1994) described that block by depolarization is a real phenomenon, but it occurs in its purest from only in certain muscle and under certain circumstances. In many instance, the first phase of block (Phase I), which is the depolarization phase, gradually merge into Phase II which exhibits characteristics that superficially resemble those of non-depolarization block. The mechanism underlying Phase II

block remains one of the mysteries of pharmacology. The explanation of the transition to Phase II is not yet clear, receptor desensitization or ion channel block may contribute to it. Possibly, after prolonged use, the drugs begin to inhibit acetylcholine synthesis or release so that Phase II is a prejunctional rather than a postjunctional phenomenon.

Another possibility hinted by experiment of Creese and Mitchell (1981) is that the initial depolarization stimulates the activity of an electrogenic membrane pump that repolarizes the membrane despite the continued present of the depolarizing agonist. The agonist molecules, now unable to depolarize the membrane, simply act to block the receptors.

The non-depolarizing neuromuscular blocking agents

The agents that act by combining with and thereby blocking the receptors but without themselves cause opening of the associated ion channels. They are often called competitive neuromuscular blocking agents.

These agents are of two broad chemical types: (1) the mono- or bisquaternary ammonium compounds, such as tubocurarine, alcuronium, doxacurium, mivacurium and atracurium (Figure 12), or (2) steroidal compounds with one or two quaternary amines attached to

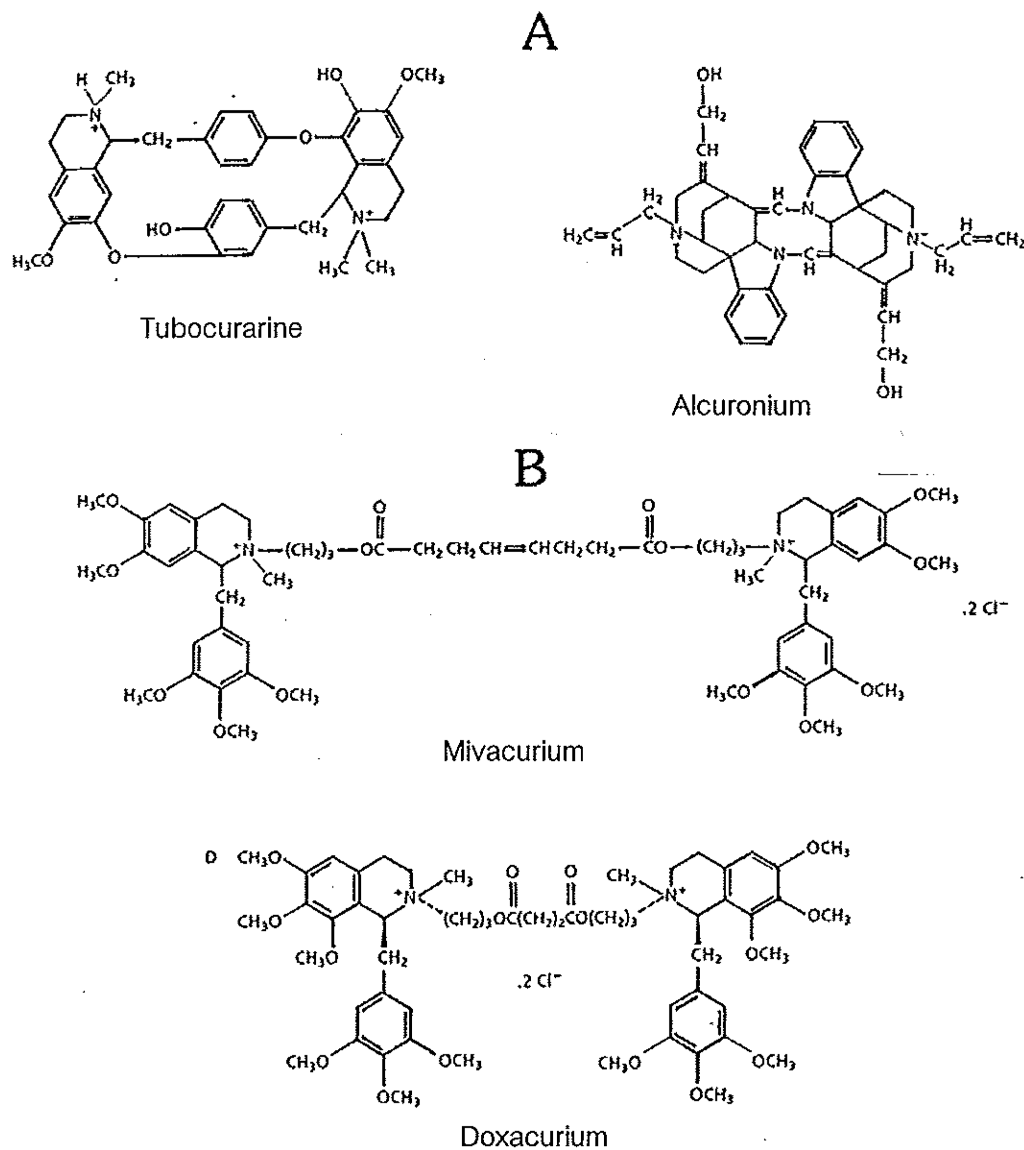
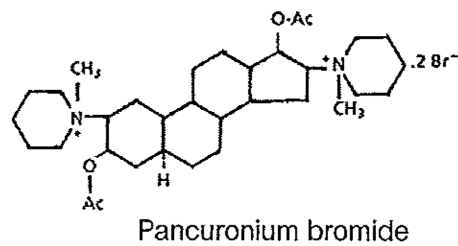


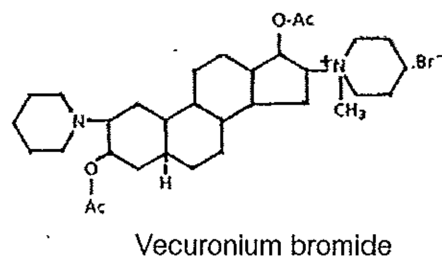
Figure 12. The chemical structure of the quaternary compounds which act as the non-depolarizing neuromuscular blocking agents.

A) The monoquaternary compounds.

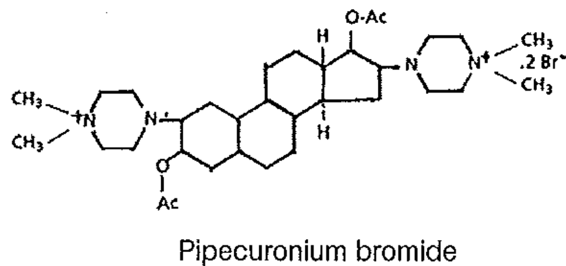
B) The bisquaternary benzylisoquinolinium compounds.



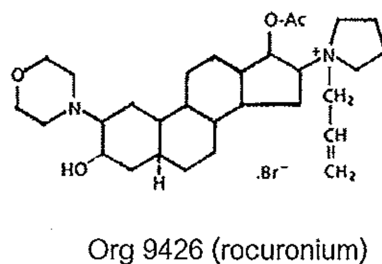
Pancuronium bromide



Vecuronium bromide



Pipecuronium bromide



Org 9426 (rocuronium)

Figure 13. The chemical structure of the steroidal non-depolarizing neuromuscular blocking agents.

the steroid nucleus, e.g. pancuronium, vecuronium, pipecuronium and rocuronium (Org 9426) (Figure 13).

Bowman (1994) reviewed about this issue in *Aneasthesia* textbook. Drugs of this type have affinity for the recognition sites of the acetylcholine receptors but are devoid of the property called "efficacy" or "intrinsic activity"; that is to say they are incapable of producing the conformational change in the receptor protein that constitutes opening of the ion channel. Hence, they act merely to impede the access of acetylcholine to the receptors. Occupation of only one of the two binding sites on a receptor complex by a blocking drug is sufficient to reduce the probability that acetylcholine will open the ion channel, since acetylcholine is most effective when it interacts with binding sites. In the presence of both acetylcholine and blocking drug, there is neither change produced by the blocking drug in the individual channel open times nor in the shape or size of the elementary currents, but the frequency of channel opening is diminished. Consequently, the sum of the elementary events constituting the endplate current (EPC) is reduced the amplitude (but not change in shape), and the reduced endplate potential (EPP) may fail to reach the threshold necessary to trigger the action potential and subsequent contraction result in muscle paralysis.

If the molecules of blocking drugs react dynamically with the receptor recognition sites, associating with and dissociating from them

repeatedly, then the block may be overcome by raising the concentration of acetylcholine and thereby enhancing its ability to complete. Tubocurarine is a classical example of reversible competitive antagonist, and certainly its effect can be overcome (within limits) by increasing the concentration of acetylcholine, e.g. with neostigmine. Other drugs of the tubocurarine type include metocurine, alcuronium, gallamine, atracurium, mivacurium and rocuronium. Alpha- bungarotoxin, on the other hand, binds irreversible to the recognition sites and so cannot be overcome by excess acetylcholine.

Antagonism of neuromuscular blockade

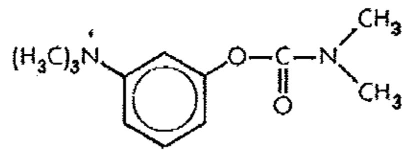
Since the non-depolarizing neuromuscular blocking agents block neuromuscular transmission predominantly by competitive antagonism of acetylcholine at the postjunctional receptor. Therefore, antagonists of non-depolarizing neuromuscular blocking agents should act by increasing the concentration of transmitter acetylcholine in the junctional cleft of the neuromuscular junction, so that competition with the blocking agent is shifted in favour of the transmitter and transmission is thereby restored. The concentration of junctional acetylcholine may be increased in two ways (Bowman, 1994):

1. By inhibiting its breakdown with anticholinesterase agents.
2. By increasing its evoked release from the nerve endings.

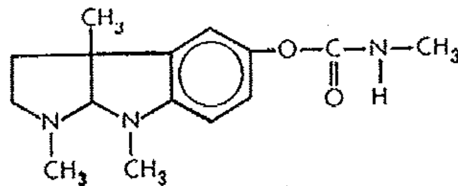
Anticholinesterase agents

Anticholinesterase agents of diverse chemical types (carbamates, analinium ions, oxamides, organophosphorus compounds, Figure 14) produce essentially the same effects. The value of particular members in the aneasthetic practice (neostigmine, pyridostigmine and edrophonium are the main ones) generally being determined more by differences in their pharmacokinetics and metabolism than by any minor differences in mechanism of action. There is suggested that the anticholinesterase agents possess facilitatory actions on neuromuscular transmission in addition to their cholinesterase inhibiting action. Neostigmine, for example, has a weak potassium channel blocking action which enhances acetylcholine release in addition to its powerful anticholinesterase action (Braga, *et al.*, 1993)

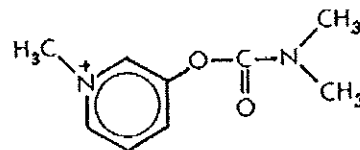
Bowman (1994) described that, normally, a single acetylcholine molecule interacts with only one receptor, or perhaps two receptors, before it collides with a cholinesterase molecule and is destroyed. However, if the junctional cholinesterase is inhibited by anticholinesterase drug, each acetylcholine molecule is able to make multiple interactions with receptors before escaping by diffusion from the junctional cleft. The consequence is that the preserved



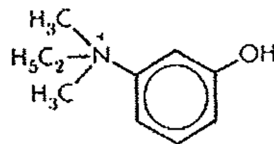
Neostigmine



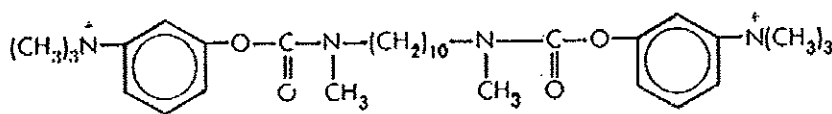
Physostigmine



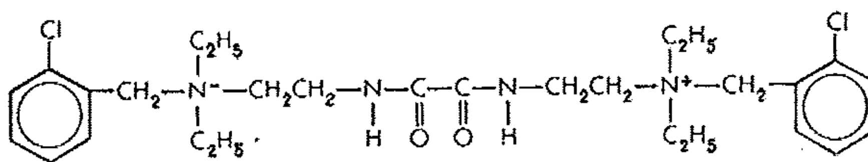
Pyridostigmine



Edrophonium



Demecorium



Ambenonium

Figure 14. The chemical structure of some anticholinesterase agents.

acetylcholine molecule has an improved chance of combining with an unblocked postjunctional receptor, so that the block is reversed. Likewise at the postulated prejunctional receptors, the preserved transmitter is able to receptor-establish the facilitatory effect on mobilization, so that the "fade" is abolished.

Substances that enhance acetylcholine release

Tetraethylammonium (TEA) is a very effective anticurare drug in cat gastrocnemius muscle preparation, indeed, TEA can antagonize the twitch depressant action of d-tubocurarine whose effect can not be antagonized by a potent anticurare drug like neostigmine (Kensler, 1950). TEA also antagonizes the twitch depressant action of d-tubocurarine in the rat phrenic nerve-diaphragm preparation (Stovner, 1958b). On the basis of the ability of TEA to increase amplitude of curarized EPP without affecting time course, and taking its negligible anticholinesterase activity into account, Stovner (1958b) postulated that TEA acts presynaptically to increase transmitter release by the nerve impulse.

Lundh and Thesleff, (1977) described that drugs that block potassium channels, such as the tetraethylamomium (TEA) and 4-aminopyridine, slow down the repolarization of the activated nerve terminal membrane, and thereby prolong to open the voltage–dependent calcium channels. Consequently, an increase influx of

calcium ions was occurred and this greatly enhances the output of neurotransmitters including acetylcholine. The effect is sufficiently powerful for the inward calcium current become clearly evident in electrophysiological experiments. The 4-aminopyridine penetrates the axonal membrane and blocks the potassium channels from the inside, whereas tetraethylammonium acts from the outside.

The ability of drugs that block potassium channels to restore normal transmission during a neuromuscular block is limited, and the "fade" is not antagonized. Indeed, they may enhance the "fade" despite an increase in initial amplitude of contraction (Gibb, *et al.*, 1982). This probably is due to the initial enhanced release of transmitter and causes temporary depletion of the readily releasable store, and then the mobilization mechanism is blocked by the neuromuscular blocking agents, therefore, the store dose not refill fast enough to match the demand (Bowman, 1986).

CHAPTER 3

MATERIALS AND METHODS

Materials

1. Experimental Animals

Experiments were performed on both sexes of adult Wistar rats weighing 200-300 gm. They were grown in the animal house of Faculty of Science, Prince of Songkla University. They were kept in animals' room of Department of Pharmacology; Faculty of Science, Prince of Songkla University at 23-25 ^{o}C with 12 hours dark/light cycles for a week before used. They were allowed to feed with standard chow pellets and tap water *ad libitum*.

2. Plant Material and Preparation

The fresh leaves of *Piper sarmentosum* Roxb. (10 kg) were obtained from Promkeeree District, Nakhorn Si Thamarat Province, Thailand, in May 1996. The plant material was identified by a botanist, Botany Section, Department of Biology, Prince of Songkla University. They were cleaned with tap water and distilled water, respectively. They were dried by air-dried. The fresh leaves 10 kg give 2 kg air-dried leaves. The dried leaves were pulverized to coarse powder

using an electric blender. The powder was extracted according to the technique used in chemical extraction which was kindly advised by Dr. Kan Chantraprомma (Chantraproma, 1983) as described below.

3. Extraction Procedure

Piper sarmentosum Roxb. leaves powder was extracted using cold extraction by macerating in 20 liters of methanol for 7 days at room temperature, and repeated for 4 times. Then, the total extract was filtered through No.1 Whatman® filter paper with suction. After filtration, the extract was evaporated at 40-45 °C by Rotavac Evaporator (Buchi®) under reduced pressure condition, at about 300 mbar, which produced by B169 Water Pump (Buchi®). The evaporated extract gave the 169.4 ml black viscous, oil-liked mixture. It was then extracted by several times of *n*-hexane to remove chlorophyll and other hexane-soluble compounds. It produced two layers; upper layer was hexane-soluble compounds and lower layer was the methanol extract. The methanol extract layer was evaporated to give 137.2 ml dark brown viscous, oil-liked mixture which was then extracted with dichloromethane : methanol : distilled water (6 : 4 : 1). Additional water was added as necessary to separate the layers. The methanol extract layer was evaporated to give 520 ml brown solution. The brown solution was freeze-dried to give 36.8 g brown powder (1.84% w/w). This methanol extract of *Piper sarmentosum* Roxb. (ME)

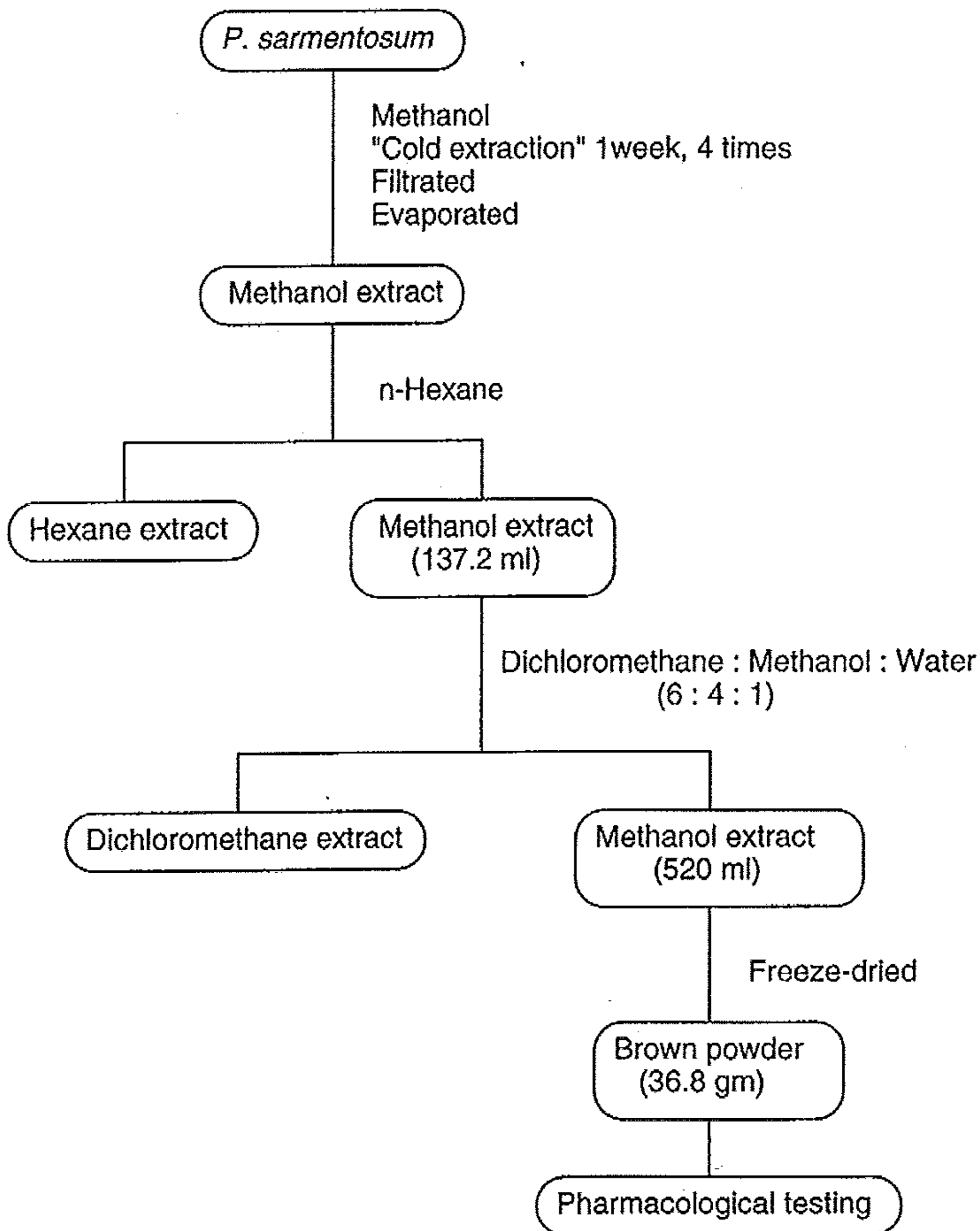


Figure 15. The flow chart shown for extracting processes to obtain the methanol extract of *Piper sarmentosum* (ME).

was stored in a bottle and kept away from moisture in a desiccator containing a moisture absorbent substance. Then, it was kept in a refrigerator at below 4 ^{o}C. The diagram of extract was shown in Figure 15.

4. Drugs and Solutions.

All experiments were performed in Krebs' solution of the following composition (Prior, *et al.*, 1995):

NaCl	118	mM
KCl	5	mM
KH_2PO_4	1.2	mM
$MgSO_4$	1	mM
$NaHCO_3$	25	mM
Glucose	11	mM
$CaCl_2$	2.5	mM

Krebs' solution was aerated with 95% O_2 and 5% CO_2 gas mixture to a pH 7.2-7.4.

The ME was freshly dissolved in Krebs' solution at the concentration of 400 mg/ml and centrifuged for 30 minutes at 2500 rpm. (Sunbhanich, *et al.*, 1988). The supernatant was used to test pharmacological activities.

The standard drugs used were the classical non-depolarizing neuromuscular blocking drug; i.e., d-tubocurarine (dTC) and succinylcholine (SCh) for depolarizing neuromuscular blocking drug.

The reversible anti-acetylcholinesterase, neostigmine methylsulphate and the drug acting on excitable cell i.e. tetraethylammonium (TEA), were used in this study.

All drugs were freshly dissolved in Krebs' solution.

Methods

1. Isolation of Rat Phrenic Nerve-Hemidiaphragm for Recording Neurally-Evoked Twitch

The phrenic nerve hemidiaphragm preparations were prepared and set up based on the modification of the techniques described by Bulbring (1946).

Rat was anaesthetized by cervical dislocation and sacrificed by decapitation, then clear off the blood from thorax by left to bleed as much as possible. The chest was open by cutting along the rib, both right and left fan-shaped hemidiaphragm accompanied by their phrenic nerve were dissected out. The preparation was then mounted in a double-walled organ bath containing 50 ml Krebs' solution. The coastal end of the hemidiaphragm was fixed with glass hook at the bottom of the organ bath and the tendon was attached to a Grass® FT 03 or FT 10 force displacement transducer (Grass Instrument Co., Quincy, Mass., USA) which was connected to a Grass® 7H polygraph (Grass Instrument Co., Quincy, Mass., USA) for recording twitch tension. The phrenic nerve was gently drawn through the loop of a platinum-wired bipolar stimulating electrode. The solution in the bath was maintained at 32 ^{o}C by circulating water from a thermostatically controlled water tank and aerated with 95% O_2 and 5% CO_2 through a needle at the bottom. The resting tension of the hemidiaphragm muscle was set at 2 gm. The preparation was equilibrated in organ

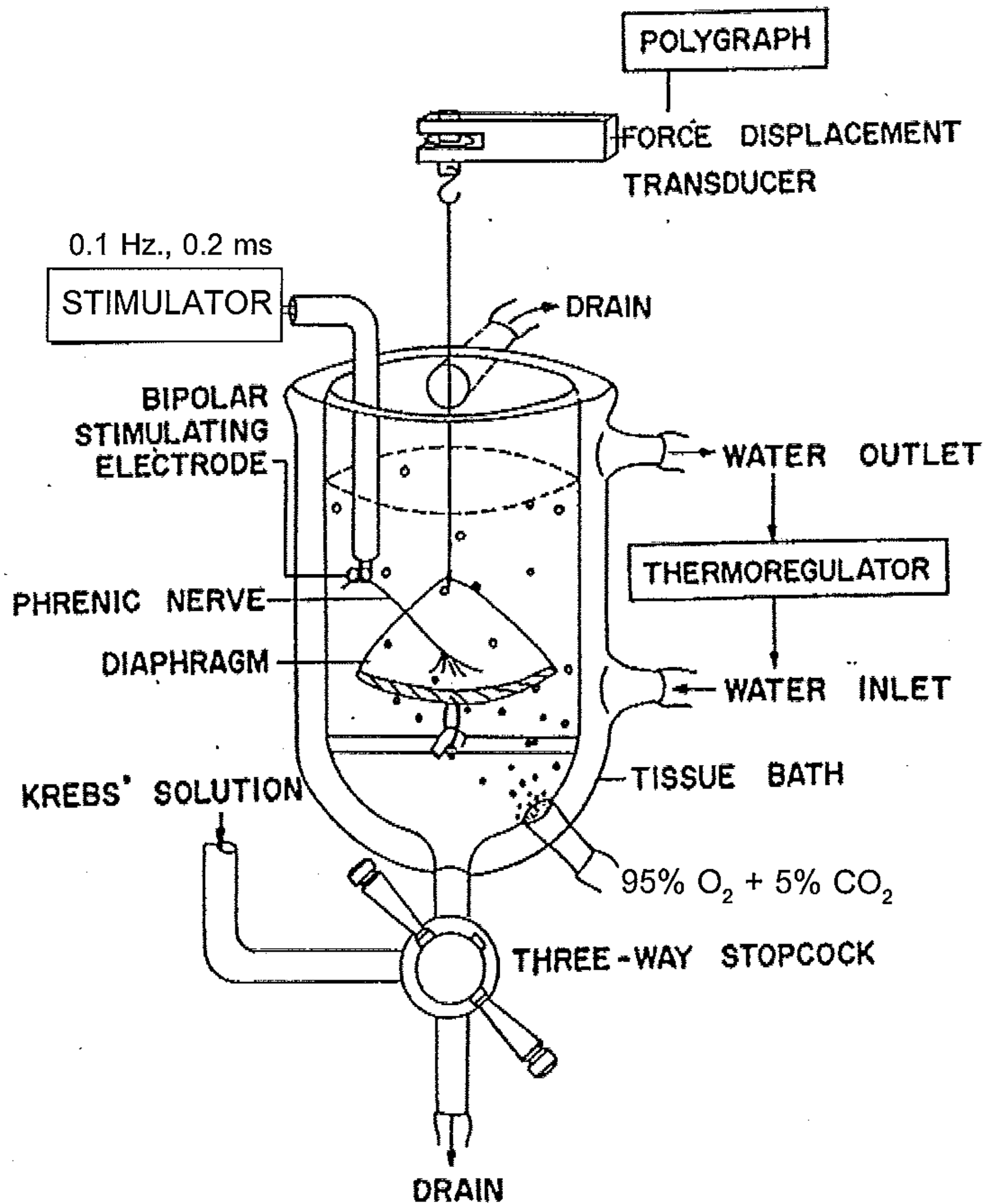


Figure 16. The set up for recording the neurally-evoked twitch in the isolated rat phrenic nerve-hemidiaphragm preparation.

bath for 30 minutes before starting the experiments. The set up for recording neurally-evoked twitch in isolated rat phrenic nerve-hemidiaphragm was illustrated in Figure 16.

The preparation was stimulated by a Grass® S 88 stimulator accompanied by Grass® SIU 5 stimulus isolation unit with supramaximal voltage square ware pulses of 0.1 Hz and 0.2 ms duration and twitch tension remained stable for 10 minutes before any study commenced.

The drugs or tested solutions in a single dose of final bath concentration was added into the organ bath to study the effects on contractile responses. The twitch tension was recorded the responses was stable or 60 minutes, then the preparation was rested at least 30 minutes and wash every 5 minutes. Each preparation was used no more than 2 different experiments.

2. *Isolation of the Rat Phrenic Nerve-Hemidiaphragm for Recording Directly-Evoked Twitch*

The preparation was set up as previously mentioned as for that of isolation of hemidiaphragm. In addition, one end of a platinum wire bipolar stimulating electrode was sutured into the muscle near the costal margin and another one was attached to the base of hemidiaphragm. The preparation was then mounted in a double-walled organ bath containing 50 ml of Krebs' solution, aerated with

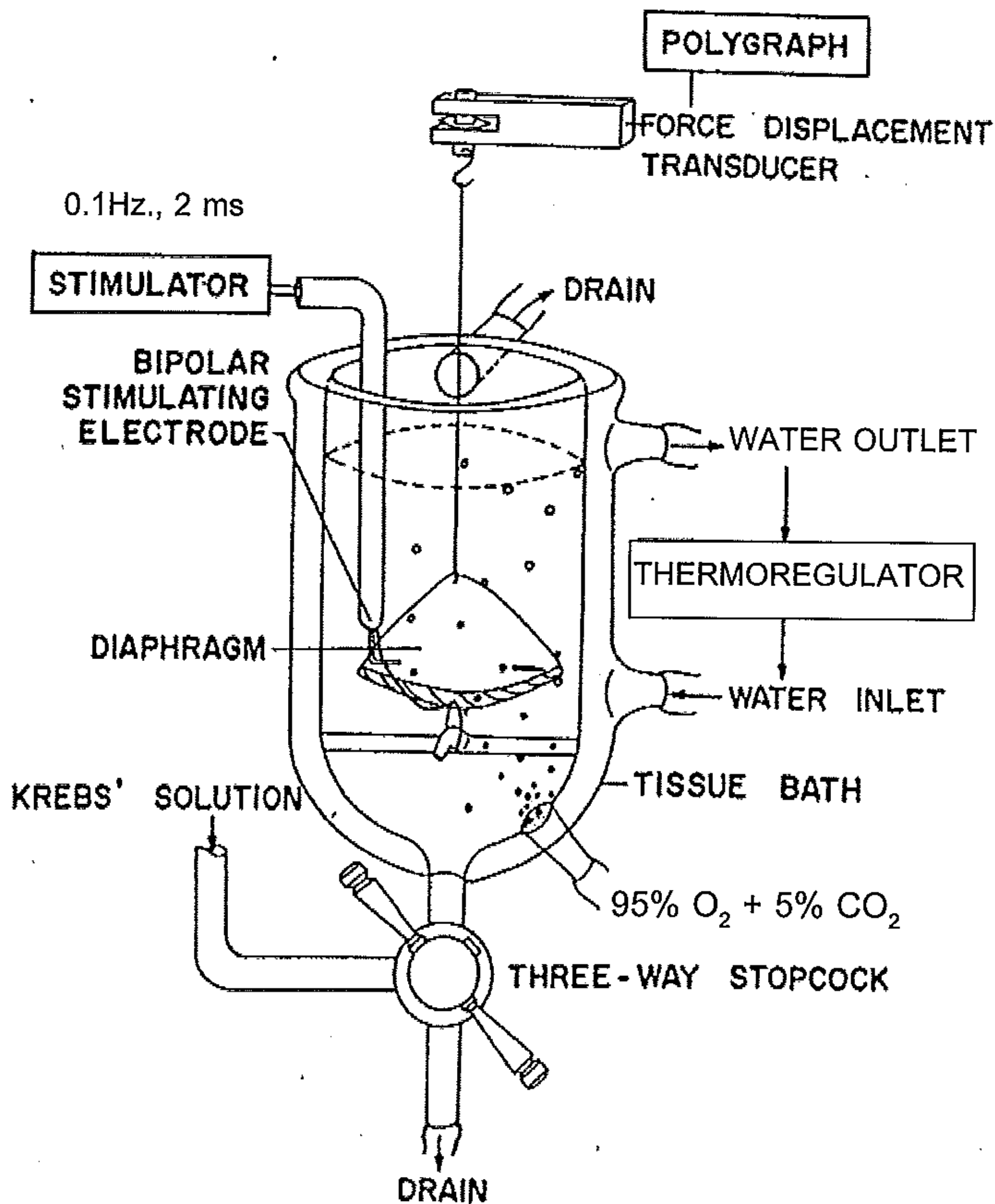


Figure 17. The set up for recording the directly-evoked twitch in the isolated rat phrenic nerve-hemidiaphragm preparation.

95% O_2 and 5% CO_2 gas mixture. The temperature was maintained at 32 °C by a thermoregulator. To eliminate the neuromuscular transmission. the preparation was completely blocked by the addition of 5 μM d-tubocurarine (Apisariyakul, 1982) into an organ bath 5 minutes before starting of the direct stimulation. Thus, this contractile response was only due to diaphragm muscle fibers. The set up for recording directly-evoked twitch in isolated rat phrenic nerve-hemidiaphragm was illustrated in Figure 17.

The preparation was stimulated by a Grass® S 88 stimulator with Grass® SIU 5 stimulus isolation unit with supramaximal voltage square ware pulses of 0.1 Hz and 2 ms duration and twitch tension remained stable for 10 minutes before any study commenced.

ME at a single dose of final bath concentration was added into the organ bath to study the effects on contractile responses. The twitch tensions were recorded until steady state or 60 minutes, then the preparation was rested for at least 30 minutes and washed every 5 minutes. Each preparation was used no more than 2 different experiments.

3. Isolation of the Rat Sciatic Nerve for Recording Action Potentials

The stimulus-evoked action potentials were recorded in the isolated rat sciatic nerve. The method was base on the modification of the technique described by Lilleheil (1970).

The rats were anesthetized with pentobarbital sodium (Nembutal®) at a dose of 50 mg/kg body weight by intraperitoneal injection. Both right and left sciatic nerves were dissected out as long as possible and transferred to a glass dish containing Krebs' solution aerated with 95% O_2 and 5% CO_2 gas mixture which the temperature was regulated at 32 °C. The nerve was mounted in a three compartment nerve chamber. The central compartment, 1 ml in volume, containing Krebs' or test solution. Both right and left compartments of the central compartment were filled with liquid paraffin. The proximal end of the sciatic nerve was placed on a pair of platinum-wired stimulating electrode in the adjacent side of central compartment and the distal end was placed on a pair of platinum-wired recording electrode in the opposite compartment. The distal end of the nerve was crushed in order to get a monophasic pattern of action potential. The nerve was constantly stimulated at a 0.1 Hz. and a rectangular pulse of 2 ms duration and supramaximal voltage.

The recording was performed on Scope™, MacLab™ application program (ADInstruments Pty Ltd., Australia), running on Macintosh Operating System.

The nerve was left in the three compartment nerve chamber for 10 minutes in order to reach an equilibrium prior to add the test solution. The stimulus-evoked action potential was observed during 30 minutes. The test solution was added to the central compartment of

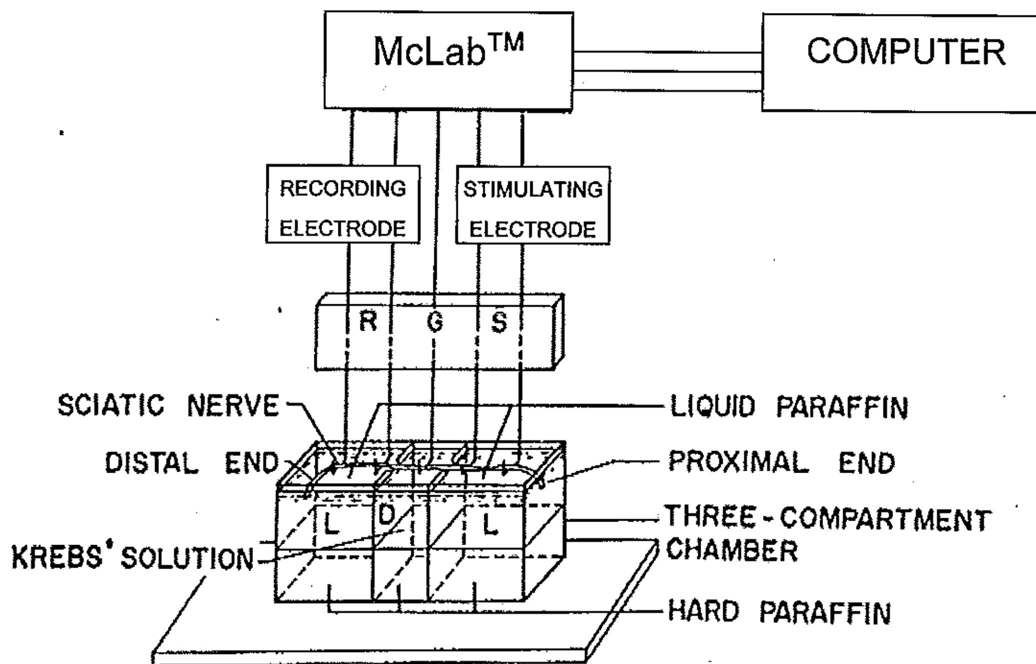


Figure 18. The set up for recording the nerve action potential on the isolated rat sciatic nerve preparation.

the nerve chamber and observed the action potential change about 30 minutes after adding the test solution. The set up for recording action potential in isolated rat sciatic nerve was illustrated in Figure 18.

4. Determination of Inorganic Ions in the ME

The concentrations of Na^{+}, K^{+} and Ca^{2+} in ME (400 mg/ml) were measured by method of Inductively Coupled Plasma atomic emission spectroscopy (ICPaes) described by Varma (1991).

5. Data Analysis

In each experiment, the twitch tension in the presence of each drug and ME were expressed as a percentage of their respective control values.

All data are presented as mean and standard error (Mean ± SE) of values from 6-8 individual experiments. The differences between sets of data were tested by a two-tailed paired Student's *t* test or analysis of variance (ANOVA) followed by Duncan multiple range test with the level of significance at $P < 0.05$.

The twitch depression or twitch tension was expressed as a percentage as follows.

$$\text{Twitch depression (\% Control)} = \frac{Tcon - Tblk}{Tcon} \times 100$$

$$\text{Twitch tension (\% Control)} = \frac{Tblk}{Tcon} \times 100$$

Where T_{con} is the control twitch tension, T_{blk} is the twitch tension at maximum block.

In the reversal studies, percentage reversal of neuromuscular block was calculated using the following equation.

$$\text{\% Antagonized} = \frac{Tant - Tblk}{Tcon - Tblk} \times 100$$

Where T_{con} is the control twitch tension, T_{blk} is the twitch tension at maximum block, before addition of the reversal agent and T_{ant} is the twitch tension at maximum antagonized.

Experimental Protocol

This investigation was divided into 2 main sections.

Section 1 The Studies on Dose-response Relationships and the Sites of Action of ME in Neurally- and Directly-Evoked Twitch

Part A The Pharmacological Effects of ME on Neurally-Evoked Twitch

1. The effects of ME on neurally-evoked twitch in the rat phrenic nerve hemidiaphragm preparation.
2. Comparison of dose-response relationships of ME with standard drugs, dTC and SCh

Part B The Pharmacological Effects of ME on Directly-Evoked Twitch.

Part C Effects of K^+ which Equals to that Presents in ME (1.6, 3.2 mg/ml) on Twitch Tension.

Part D Effects of ME on the Nerve Action Potential

Section 2 Interaction of ME and Drugs or Agents effecting on Neuromuscular Junction

Part A The Synergistic Effect Studies

1. The Effect of ME in the presence of dTC
2. The Effect of ME in the presence of SCh

Part B The Antagonistic Effect Studies.

1. The antagonistic effect of ME after dTC
2. The antagonistic effect of ME after SCh
3. The antagonistic effects of neostigmine (NS) on 50% neuromuscular block induced by dTC or ME
4. The antagonistic effects of tetraethylammonium (TEA) on 50% neuromuscular block induced by dTC or ME

Part C Effect of ME on Acetylcholine Contraction

CHAPTER 4

RESULTS

The results in this investigation were divided into 2 main sections as follows :

Section 1 The Studies on Dose-response Relationships and Sites of Action of ME in Neurally and Directly-Evoked Twitch

Part A The Pharmacological Effects of ME on Neurally-Evoked Twitch

1. The effects of ME on neurally-evoked twitch in the isolated rat phrenic nerve-hemidiaphragm preparation.

The effect of ME at final concentrations of 1.6, 3.2, 4.0, 4.8 and 6.4 mg/ml on the neurally-evoked twitch were taken at 30 minutes after addition of the extract. It was found that 1.6 mg/ml of the extract produced only twitch potentiation. At higher concentrations (3.2, 4.0, 4.8 and 6.4 mg/ml), ME produced an initial twitch potentiation and followed by twitch depression. The representative tracings and time-action relationships (0-60 minutes) were illustrated in Figure 19, Table 1 and Figure 20, respectively.

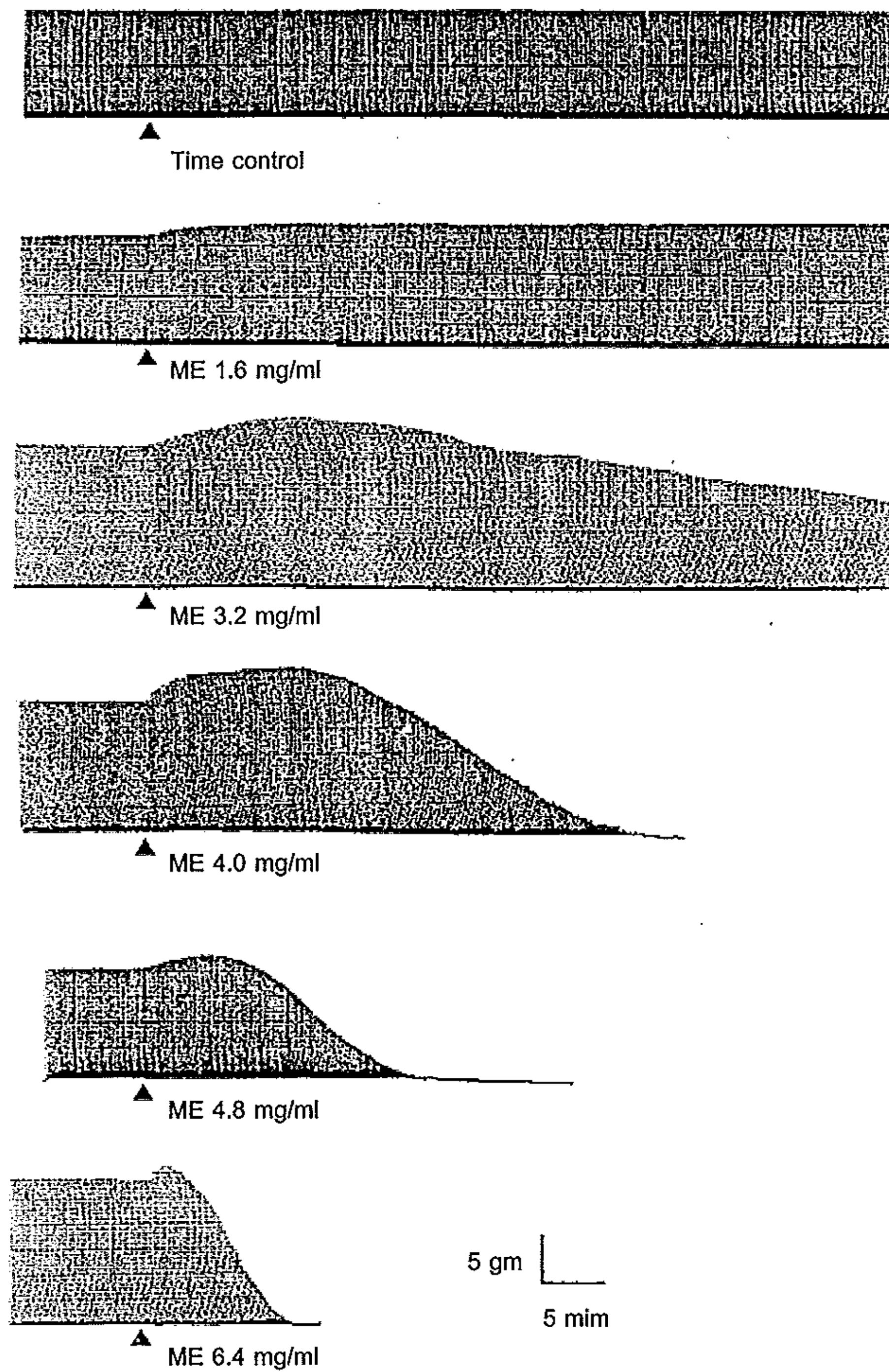


Figure 19. The representative tracings of ME on neurally-evoked twitch in the isolated rat phrenic nerve-hemidiaphragm preparation.

Table 1. The time-action relationships of ME on neurally-evoked twitch. The data were presented as mean ± SE of 8 observations.

Concentration (mg/ml)	Twitch tension (% Control)								
	0 min	2 min	5 min	10 min	20 min	30 min	40 min	50 min	60 min
1.6	100.00 ±0.00	- -	107.93 ±1.19	111.15 ±1.51	112.74 ±2.24	113.27 ±2.87	113.21 ±3.17	112.94 ±3.49	113.30 ±3.91
3.2	100.00 ±0.00	- -	110.89 ±1.897	113.47 ±3.82	107.26 ±5.80	96.20 ±6.15	84.58 ±6.02	74.85 ±5.46	66.75 ±5.85
4.0	100.00 ±0.00	- -	108.45 ±1.95	110.44 ±1.42	90.17 ±3.78	56.64 ±5.50	28.87 ±5.60	12.55 ±4.83	5.61 ±3.05
4.8	100.00 ±0.00	- -	115.61 ±2.02	107.73 ±4.39	32.51 ±4.65	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00
6.4	100.00 ±0.00	110.05 ±0.74	96.00 ±3.73	37.09 ±11.45	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00

Table 2. The twitch depression produced by ME on neurally-evoked twitch at 30 minutes. The data were presented as mean ± SE of 8 observations. ME at concentration of 1.6 mg/ml produced only an increase in twitch tension amplitude (13.27 ± 2.89 %).

Concentration (mg/ml)	Twitch depression (% Control)
1.6	0
3.2	3.80 ± 6.15
4.0	43.36 ± 5.50
4.8	100.00 ± 0.00
6.4	100.00 ± 0.00

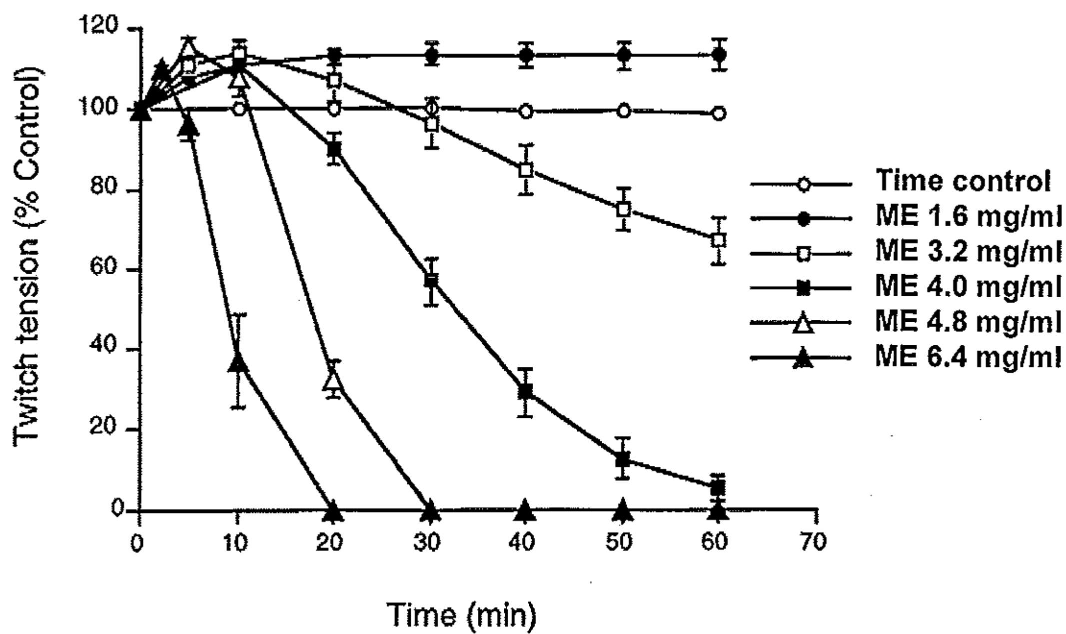


Figure 20. The time-action relationships of ME on neurally-evoked twitch for 60 minutes in the isolated rat phrenic nerve-hemidiaphragm preparation. Each point is mean ± SE of 8 observations.

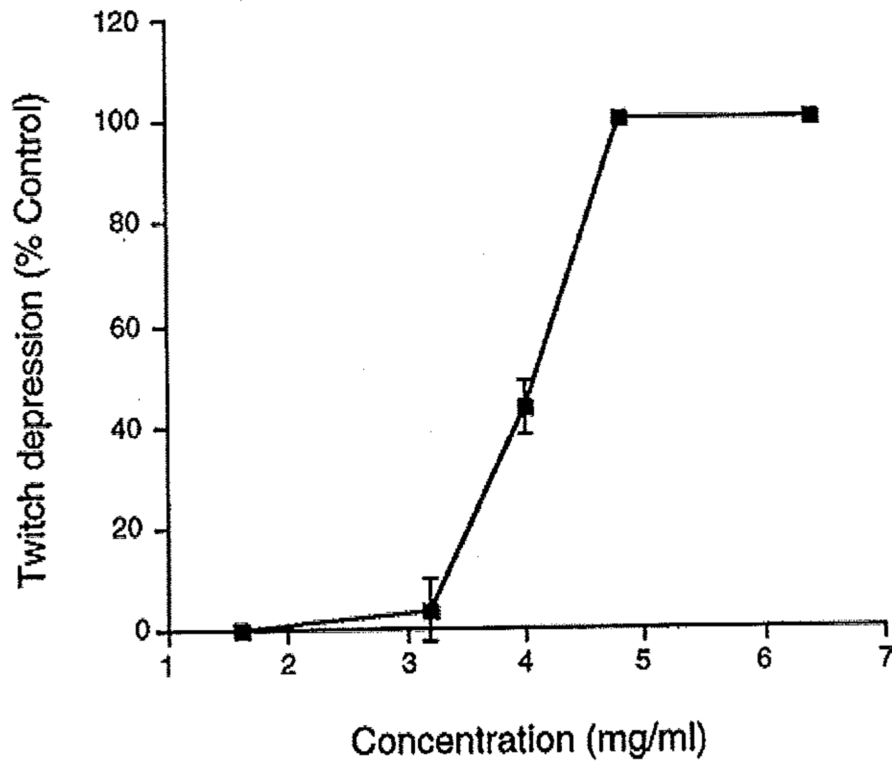


Figure 21. The dose-response curve of ME on neurally-evoked twitch at 30 minutes in the isolated rat phrenic nerve-hemidiaphragm preparation. Each point is mean ± SE of 8 observations.

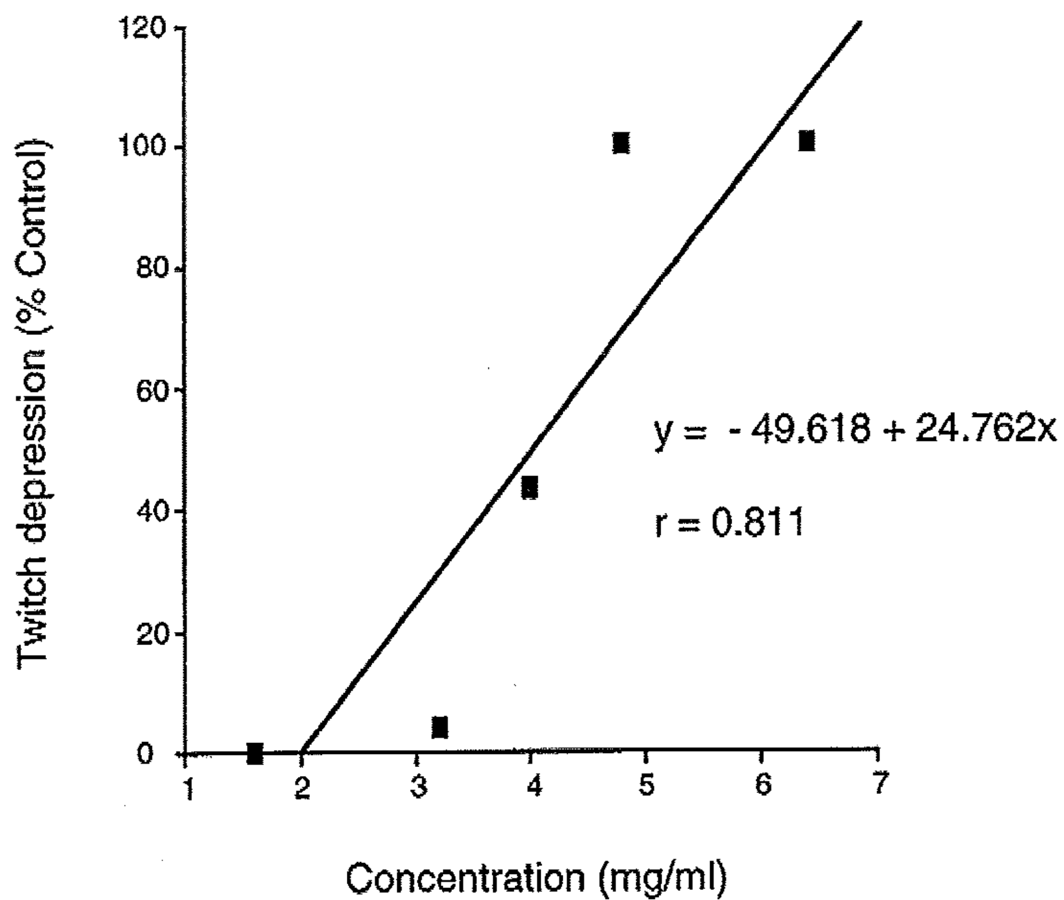


Figure 22. The regression line of ME on neurally-evoked twitch at 30 minutes in the isolated rat phrenic nerve-hemidiaphragm preparation.

The higher concentrations produced higher degree of neuromuscular blockade (Table 2). The EC_{50} for ME was 4.07 mg/ml. These effects were dose-dependent manner (r = 0.811). The dose-response curve and regression line were shown in Figure 21 and 22, respectively.

2. Comparison of dose-response relationship of ME and standard drugs, dTC and SCh.

The neurally-evoked twitch dose-response curve of ME was compared to those of standard neuromuscular blocking drugs, dTC and SCh.

dTC at final concentration of 0.5, 1.0, 1.5 and 2.0 μM were added into the organ bath to observe the effect on contractile responses which were collected at 30 minutes. It was found that all doses of dTC produced only twitch depression. The representative tracings and time-action relationships (0-60 minutes) were illustrated in Figure 23, Table 3 and Figure 24, respectively. The higher doses produced higher degree of neuromuscular blockade as shown in Table 4. The EC_{50} for dTC was 1.1 μM. These effects were dose-dependent (r=0.897). The dose-response curve and regression line were shown in Figure 25 and 26, respectively.

SCh at final concentration of 5, 10, 15, 20, 25 and 30 μM were added into the organ bath to observe the effect on contractile

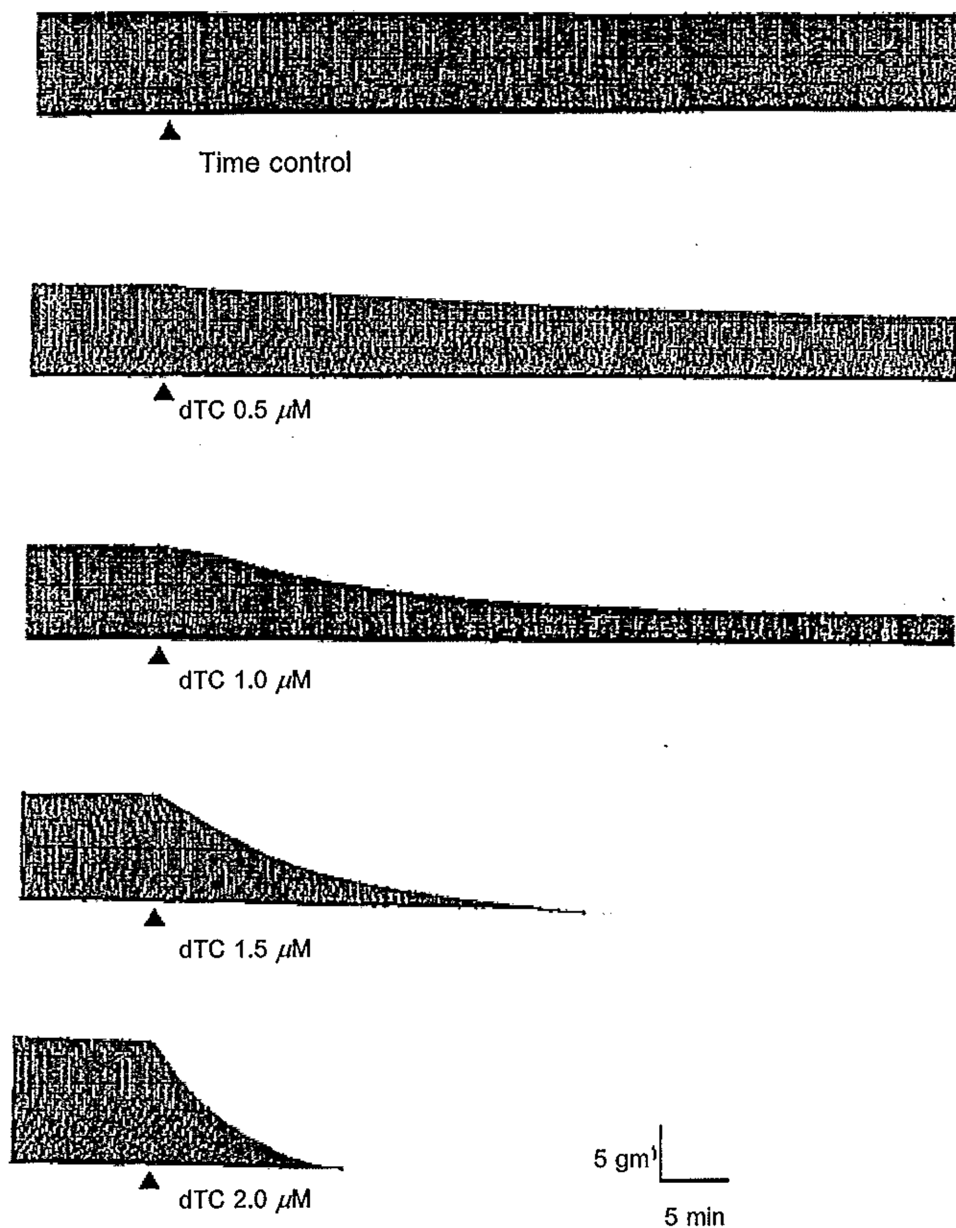


Figure 23. The representative tracings of dTC on neurally-evoked twitch in the isolated rat phrenic nerve-hemidiaphragm preparation.

Table 3. The time-action relationships of dTC. The data were presented as mean ± SE of 8 observations.

Concentration (μM)	Twitch tension (% Control)						
	0 min	10 min	20 min	30 min	40 min	50 min	60 min
0.5	100 ± 0.00	97.06 ± 0.47	94.47 ± 0.74	91.70 ± 0.77	89.03 ± 0.66	86.93 ± 0.74	85.73 ± 0.77
1.0	100.00 ± 0.00	85.08 ± 2.00	69.82 ± 2.75	60.61 ± 2.47	54.49 ± 2.63	49.82 ± 2.24	45.09 ± 2.07
1.5	100.00 ± 0.00	47.98 ± 4.84	14.95 ± 1.30	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2.0	100.00 ± 0.00	20.02 ± 2.88	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

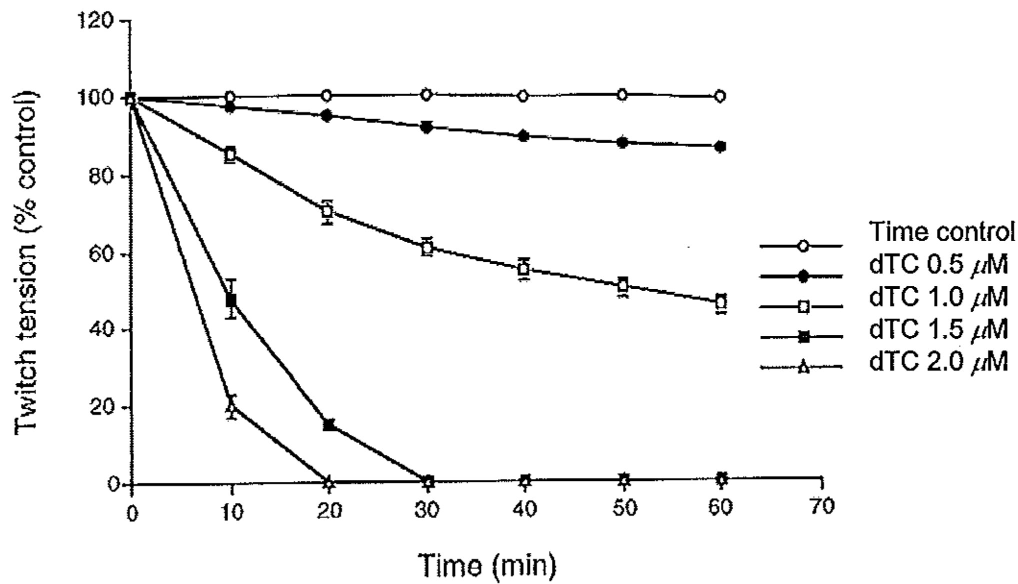


Figure 24. The time-action relationships of dTC for 60 minutes in the isolated rat phrenic nerve-hemidiaphragm preparation. Each point is mean ± SE of 8 observations

Table 4. The twitch depression produced by dTC at 30 minutes. The data were presented as mean ± SE of 8 observations.

Concentration (μM)	Twitch depression (% Control)
0.5	8.3 ± 0.77
1.0	39.39 ± 2.47
1.5	100.00 ± 0.00
2.0	100.00 ± 0.00

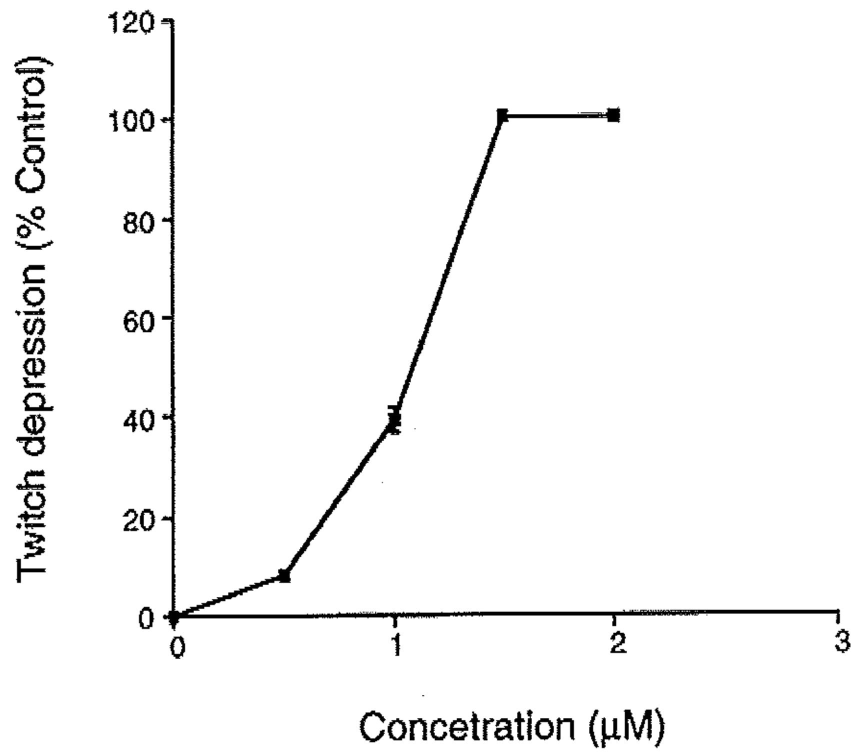


Figure 25. The dose-response curve of dTC at 30 minutes in the isolated rat phrenic nerve-hemidiaphragm preparation. Each point is mean ± SE of 8 observations.

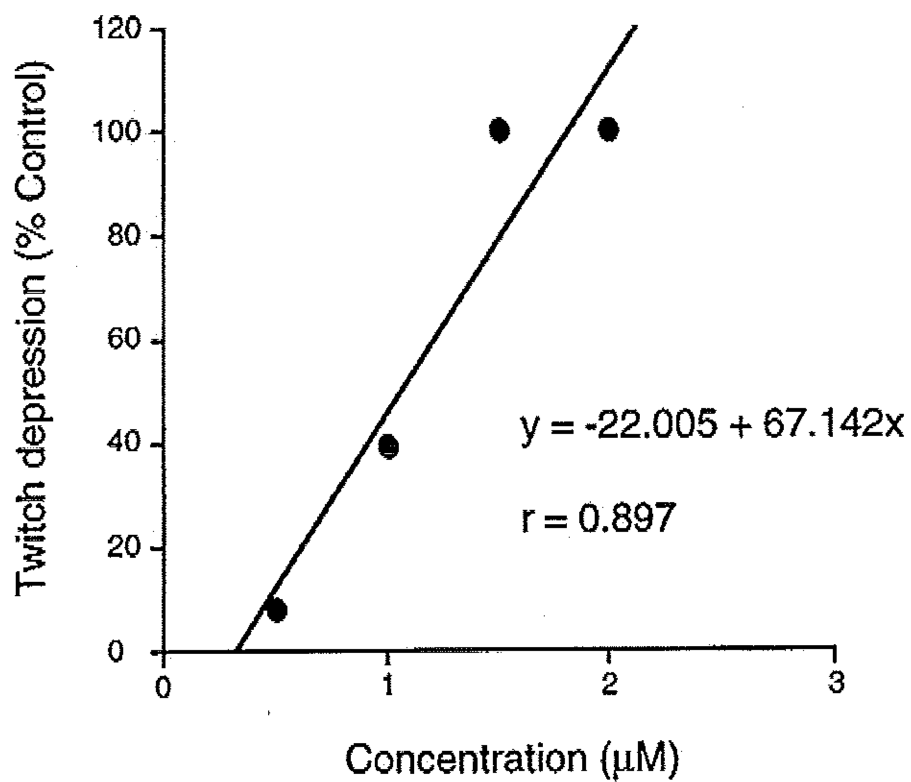


Figure 26. The regression line of dTC at 30 minutes in the isolated rat phrenic nerve-hemidiaphragm preparation.

responses which were collected at 30 minutes. It was found that low doses of SCh (5 μM) produced an initially increase (1.53 ± 0.80) and followed by slightly decrease in twitch tension (7.47 ± 1.39), others produced only a decrease in twitch tension. The representative tracings and time-action relationships (0-60 minutes) were illustrated in Figure 27, Table 5 and Figure 28, respectively.

The higher doses produced higher degree of neuromuscular blockade which shown in Table 6. The EC_{50} for SCh was 15 μM. These effects were dose-dependent (r=0.930). The dose-response curve and regression line were shown in Figure 29 and 30, respectively.

Part B The Pharmacological Effects of ME on Directly-Evoked Twitch

The isolated rat phrenic nerve hemidiaphragm preparations were performed and the preparation were completely curarized by adding 5 μM of dTC in order to prevent the branches of phrenic nerve affected by electrical stimulation, so the muscle twitch obtained was only due to electrical stimulation of muscle fibers. The ME at final concentrations of the same series which used in neurally-evoked twitch were added into the organ bath to observe the effect on contractile responses which were collected at 30 minutes.

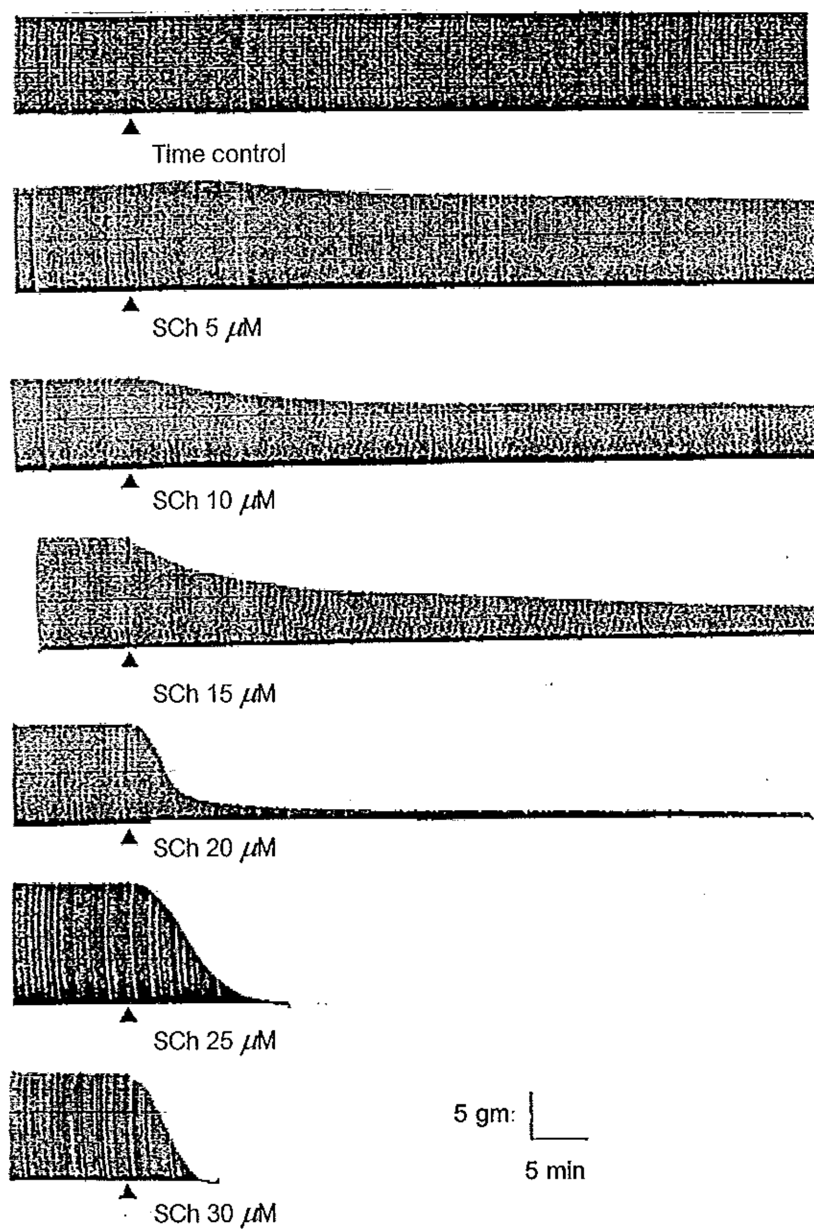


Figure 27. The representative tracings of SCh on neurally-evoked twitch in the isolated rat phrenic nerve-hemidiaphragm preparation.

Table 5. The time-action relationships of SCh. The data were presented as mean ± SE of 8 observations.

Concentration (μM)	Twitch tension (% Control)							
	0 min	5 min	10 min	20 min	30 min	40 min	50 min	60 min
5	100.00 ±0.00	101.53 ±0.80	100.20 ±0.91	95.83 ±1.14	92.53 ±1.39	89.63 ±1.65	86.68 ±1.80	84.12 ±1.99
10	100.00 ±0.00	96.51 ±1.96	89.79 ±2.44	81.63 ±2.24	77.29 ±2.19	73.22 ±2.13	70.37 ±2.34	67.28 ±2.64
15	100.00 ±0.00	87.53 ±4.70	64.98 ±5.84	50.72 ±5.74	49.38 ±4.25	47.95 ±3.82	45.47 ±3.70	43.13 ±3.76
20	100.00 ±0.00	46.18 ±5.16	22.08 ±2.66	14.45 ±2.54	11.41 ±2.14	8.45 ±2.22	5.85 ±1.90	3.85 ±1.30
25	100.00 ±0.00	32.70 ±4.03	6.27 ±0.83	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00
30	100.00 ±0.00	22.35 ±3.56	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00

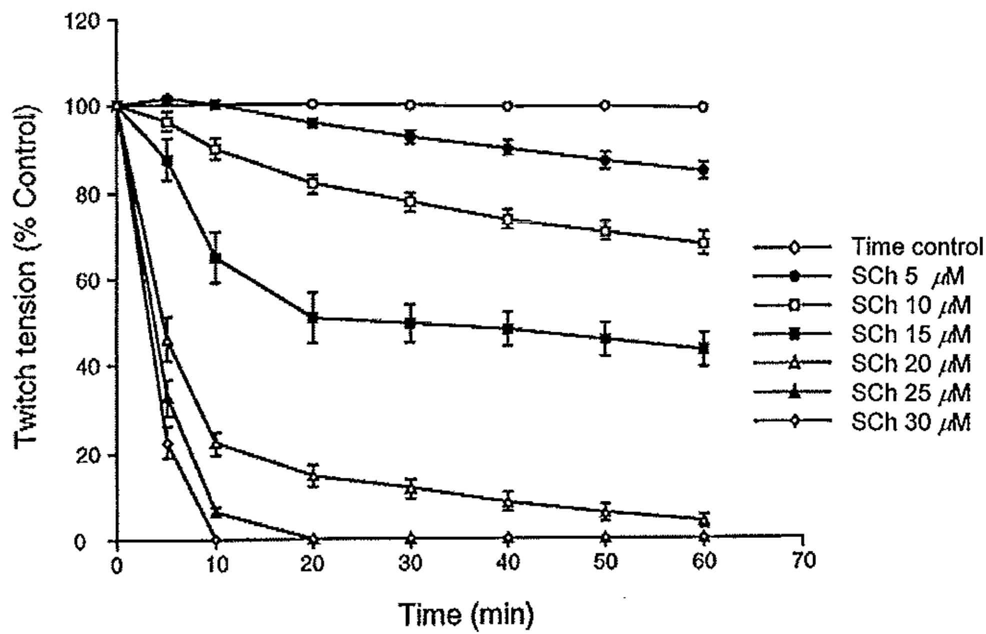


Figure 28. The time-action relationships of SCh on neurally-evoked twitch for 60 minutes in the isolated rat phrenic nerve-hemidiaphragm preparation. Each point is mean ± SE of 8 observations

Table 6. The twitch depression produced by SCh at 30 minutes. The data were presented as mean ± SE of 8 observations.

Concentration (μM)	Twitch depression (% Control)
5	7.47 ± 1.39
10	22.71 ± 2.19,
15	50.62 ± 4.25
20	88.59 ± 2.14
25	100.00 ± 0.00
30	100.00 ± 0.00

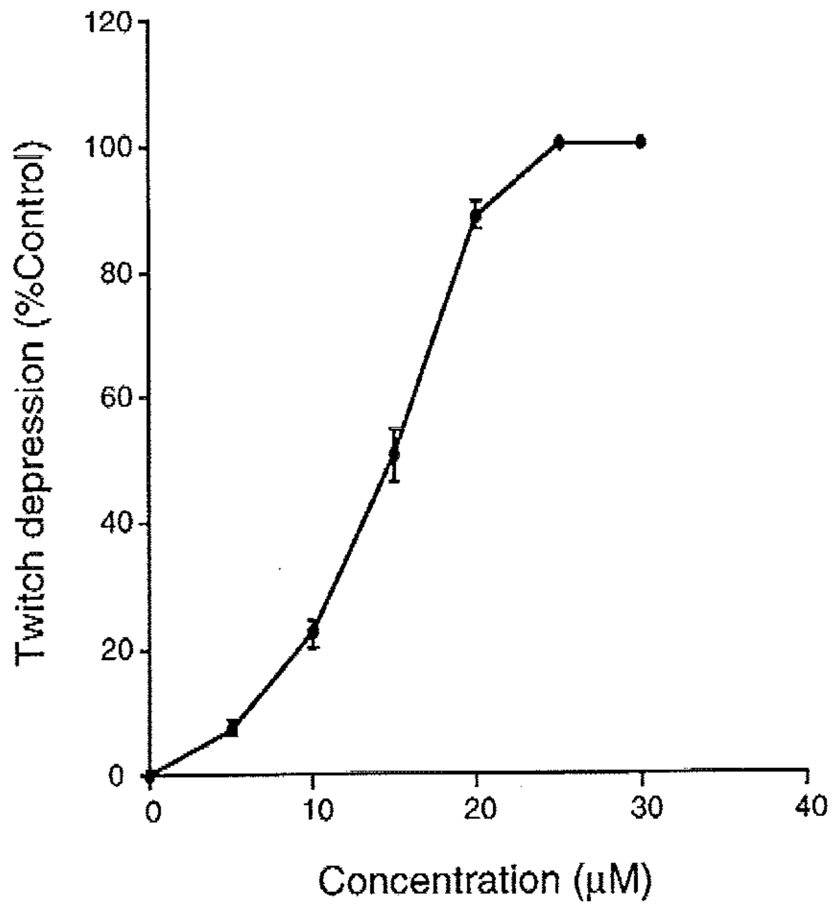


Figure 29. The dose-response curve of SCh at 30 minutes in the isolated rat phrenic nerve-hemidiaphragm preparation. Each point is mean ± SE of 8 observations.

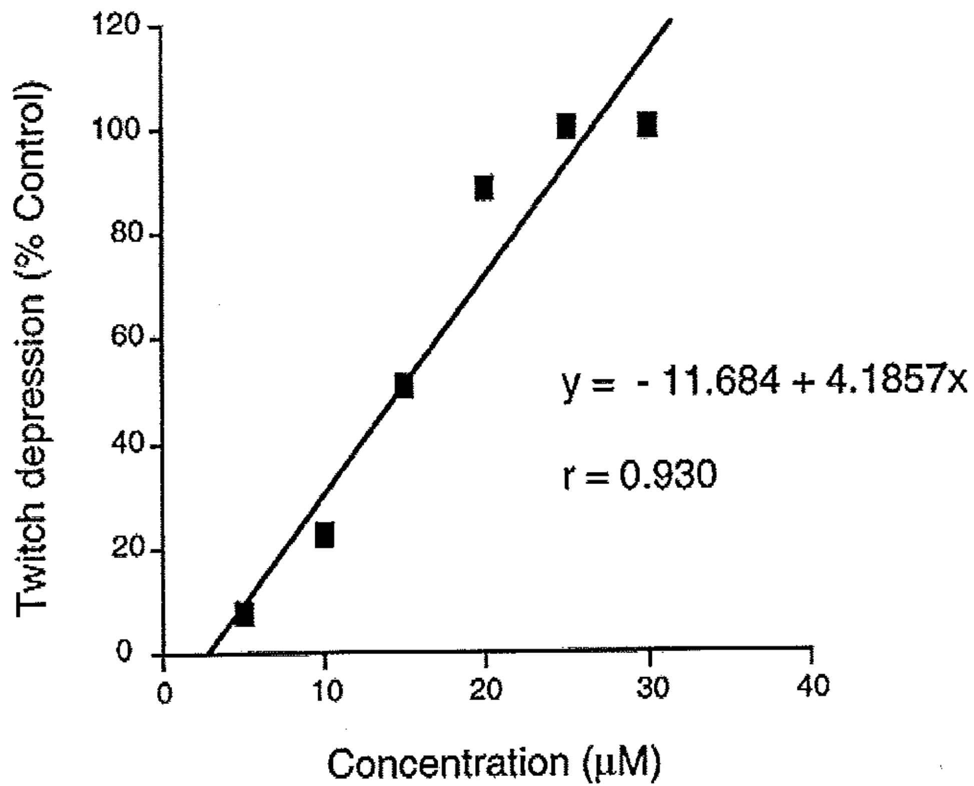


Figure 30. The regression line of SCh at 30 minutes in the isolated rat phrenic nerve-hemidiaphragm preparation.

It was found that 1.6, 3.2, and 4.0 mg/ml of the extract produced only an increase in twitch contraction amplitude. The higher concentrations, 4.8 and 6.4 mg/ml produced an initial twitch potentiation and followed by twitch depression. The representative tracings and time-action relationships (0-60 minutes) were illustrated in Figure 31, Table 7 and Figure 32, respectively. The EC_{50} for ME on directly-evoked twitch was 5.0 mg/ml. These effects were dose-dependent (r=0.749). The dose-response curve and regression line were shown in Table 8, Figure 33 and 34, respectively.

The dose-response curve of ME on neurally-evoked twitch was compared to the dose-response curve obtained from directly-evoked twitch as shown in Table 9 and Figure 35 and found that depressive effect of ME on neurally-evoked twitch was more potent than occurring on directly-evoked twitch.

Part C Effects of K^+ which Equals to that Presents in ME (1.6, 3.2 mg/ml) on Twitch tension

Since the ME is a crude methanol extract, it is composed of many chemicals and ions. Those ions might have an effect on neuromuscular junction. Potassium, sodium, calcium and magnesium are known to affect neuromuscular transmission. Therefore, these ions concentration in ME (400 mg/ml) was determined by method of

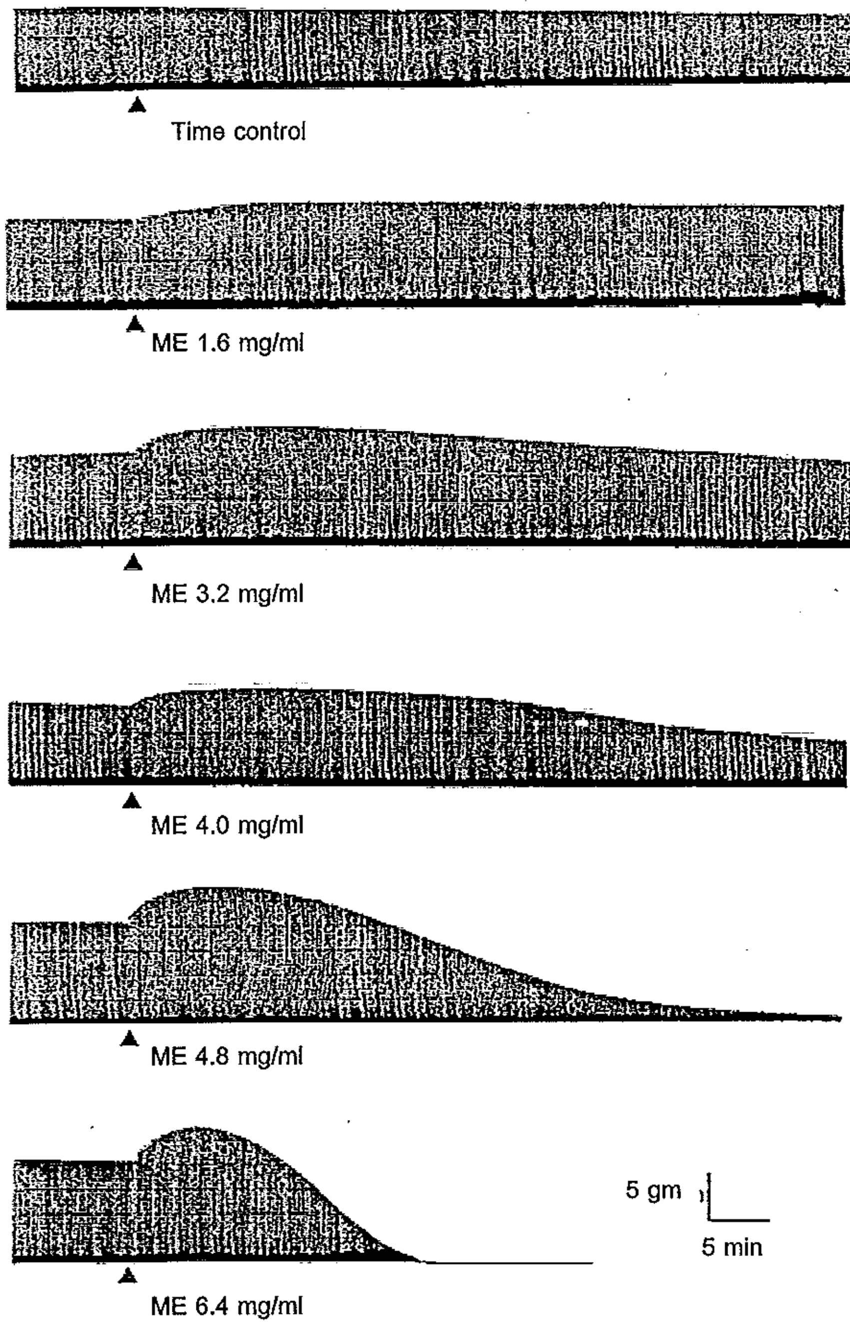


Figure 31. The representative tracings of ME on directly-evoked twitch in the curarized isolated rat phrenic nerve-hemidiaphragm preparation.

Table 7. The time-action relationships of ME on directly-evoked twitch. The data were presented as mean ± SE of 8 observations.

Concentration (mg/ml)	Twitch tension (% Control)							
	0 min	5 min	10 min	20 min	30 min	40 min	50 min	60 min
1.6	100.00 ± 0.00	- -	110.95 ± 2.26	113.27 ± 2.92	112.46 ± 2.68	111.63 ± 2.52	110.05 ± 2.67	108.64 ± 2.58
3.2	100.00 ± 0.00	- -	119.59 ± 3.48	113.82 ± 3.88	102.94 ± 2.87	93.84 ± 2.71	87.27 ± 3.12	81.46 ± 3.14
4.0	100.00 ± 0.00	- -	129.30 ± 5.33	123.58 ± 5.86	111.23 ± 6.39	98.06 ± 7.24	90.10 ± 13.41	68.39 ± 6.55
4.8	100.00 ± 0.00	126.58 ± 2.82	131.16 ± 2.75	107.51 ± 4.74	61.83 ± 5.75	22.75 ± 5.05	5.58 ± 2.51	1.23 ± 0.83
6.4	100.00 ± 0.00	136.18 ± 2.29	122.20 ± 4.55	28.38 ± 7.36	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

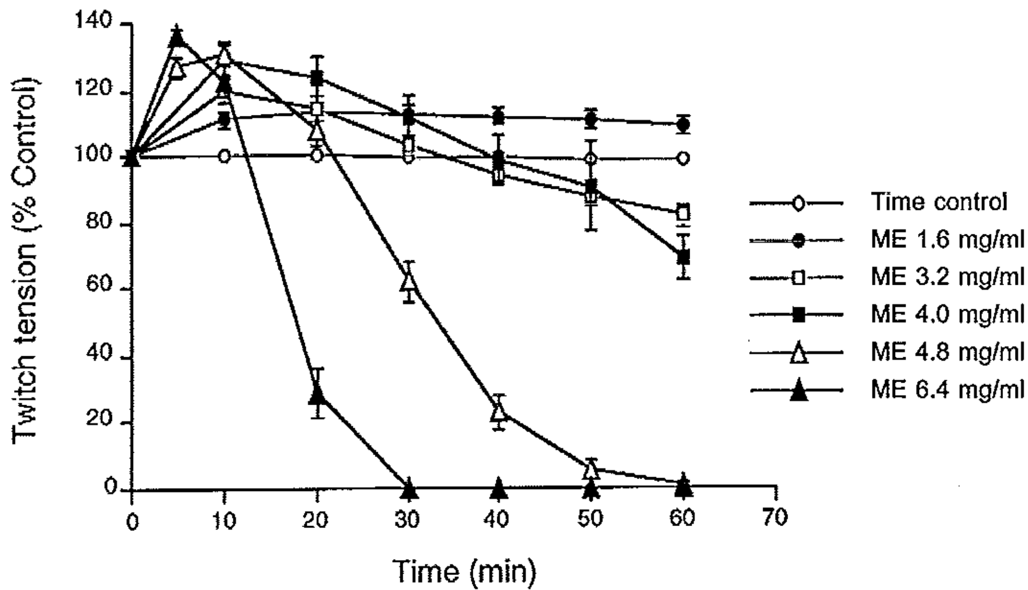


Figure 32. The time-action relationships of ME on directly-evoked twitch for 60 minutes in the curarized isolated rat phrenic nerve-hemidiaphragm preparation. Each point is mean ± SE of 8 observations.

Table 8. The twitch depression produced by ME on directly-evoked twitch at 30 minutes. The data were presented as mean ± SE of 8 observations. ME at final concentrations of 1.6, 3.2 and 4.0 mg/ml produced only an increase in twitch tension amplitude (12.46 ± 2.68, 2.94 ± 2.87, and 11.23 ± 6.39 %, respectively).

Concentration (mg/ml)	Twitch depression (% Control)
1.6	0
3.2	0
4.0	0
4.8	38.17 ± 5.75
6.4	100.00 ± 0.00

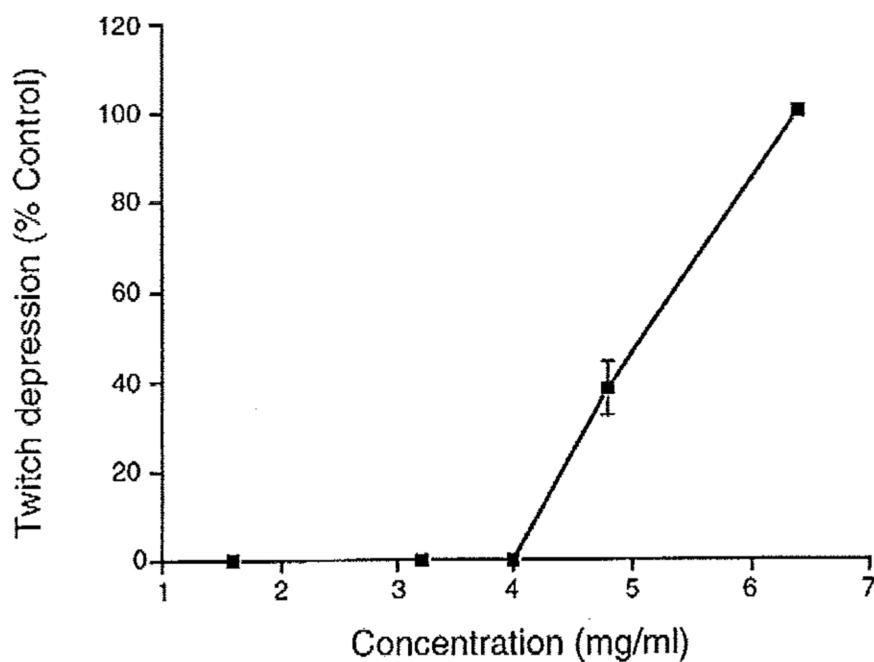


Figure 33. The dose-response curve of ME on directly-evoked twitch at 30 minutes in the curarized isolated rat phrenic nerve-hemidiaphragm preparation. Each point is mean ± SE of 8 observations.

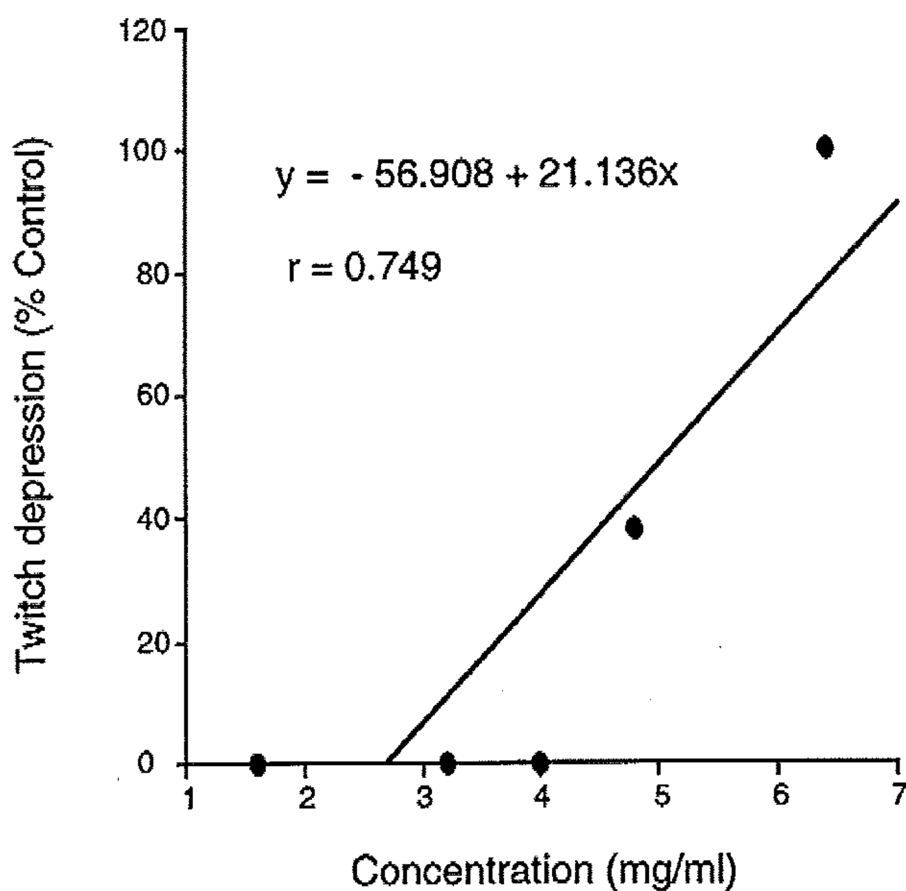


Figure 34. The regression line of ME on directly-evoked twitch at 30 minutes in the curarized isolated rat phrenic nerve-hemidiaphragm preparation.

Table 9. Comparison of dose-responses of ME on neurally- and directly-evoked twitch. The data were presented as mean ± SE of 8 and 6 observations, respectively.

Concentration	Twitch depression (% Control)	
(mg/ml)	Neurally-evoked twitch	Directly- evoked twitch
1.6	0	0
3.2	3.80 ± 6.15	0
4.0	43.36 ± 5.50	0*
4.8	100.00 ± 0.00	38.17 ± 5.75*
6.4	100.00 ± 0.00	100.00 ± 0.00
EC_{50}	4.07 mg/ml	5.00 mg/ml

Note: Asterisk (*) indicates significant difference from control ($P < 0.05$)

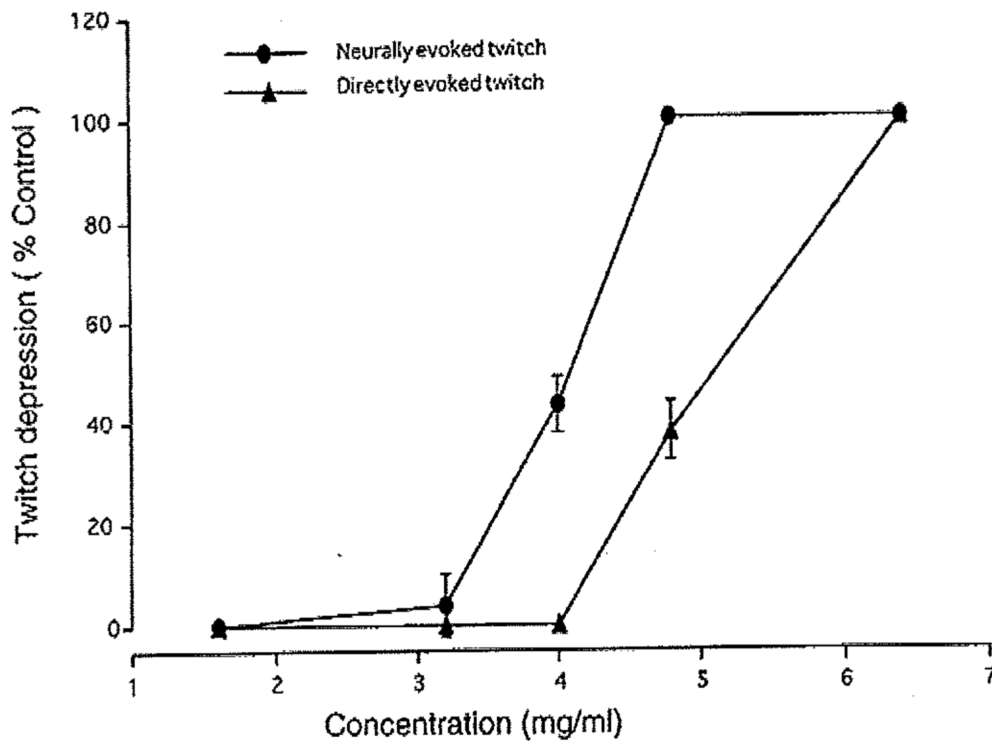


Figure 35. Comparison of dose-response curves of ME on neurally-evoked twitch and directly-evoked twitch.

Inductively Coupled Plasma Atomic Emission Spectroscopy (ICPaes; Varma, 1991). It was found that K^+ was the major ion in ME (Table 10A). The effect of K^+ was studied in comparison to the effect of ME in this preparation. The concentration of K^+ was calculated based on ME concentration that usually used in this investigation (Table 10B). Potassium chloride (KCl) at final concentrations of 3.5 and 7.0 mM, which were closely equivalent to the amount of K^+ containing in 1.6 and 3.2 mg/ml of ME, respectively, were tested on this preparation. After adding KCl solution, it was found that both concentrations of KCl produced a slight increase in twitch tension both in neurally- and directly-evoked twitch without any degree of neuromuscular blockade. The representative tracings and time-action relationships were illustrated in Figure 36 and Table 11, respectively.

Part D Effects of ME on the Nerve Action Potentials

In this part, the direct effect of ME on nerve impulse conduction was studied. Instead of the phrenic nerve, the sciatic nerves were used in this experiment, because it gave a bigger action potential.

The sciatic nerve was placed on the three-compartment chamber and the amplitude of action potential was observed every 10 minutes for 30 minutes as control. It was found that the amplitude of

Table 10. The concentration of K^+, Na^+, Ca^{2+} and Mg^{2+} in the 400 mg/ml of ME measured by the method of ICPaes (A), and in 1.6, 3.2 mg/ml of ME determined by calculation (B).

A

Ions	Concentration (mM)
K^+	878.77 ± 1.74
Na^+	174.09 ± 0.38
Ca^{2+}	2.77 ± 0.01
Mg^{2+}	8.74 ± 0.02

B

Ions	Concentration (mM)	
	ME 1.6 mg/ml	ME 3.2 mg/ml
K^+	3.525	7.030
Na^+	0.572	1.144
Ca^{2+}	0.010	0.020
Mg^{2+}	0.004	0.008

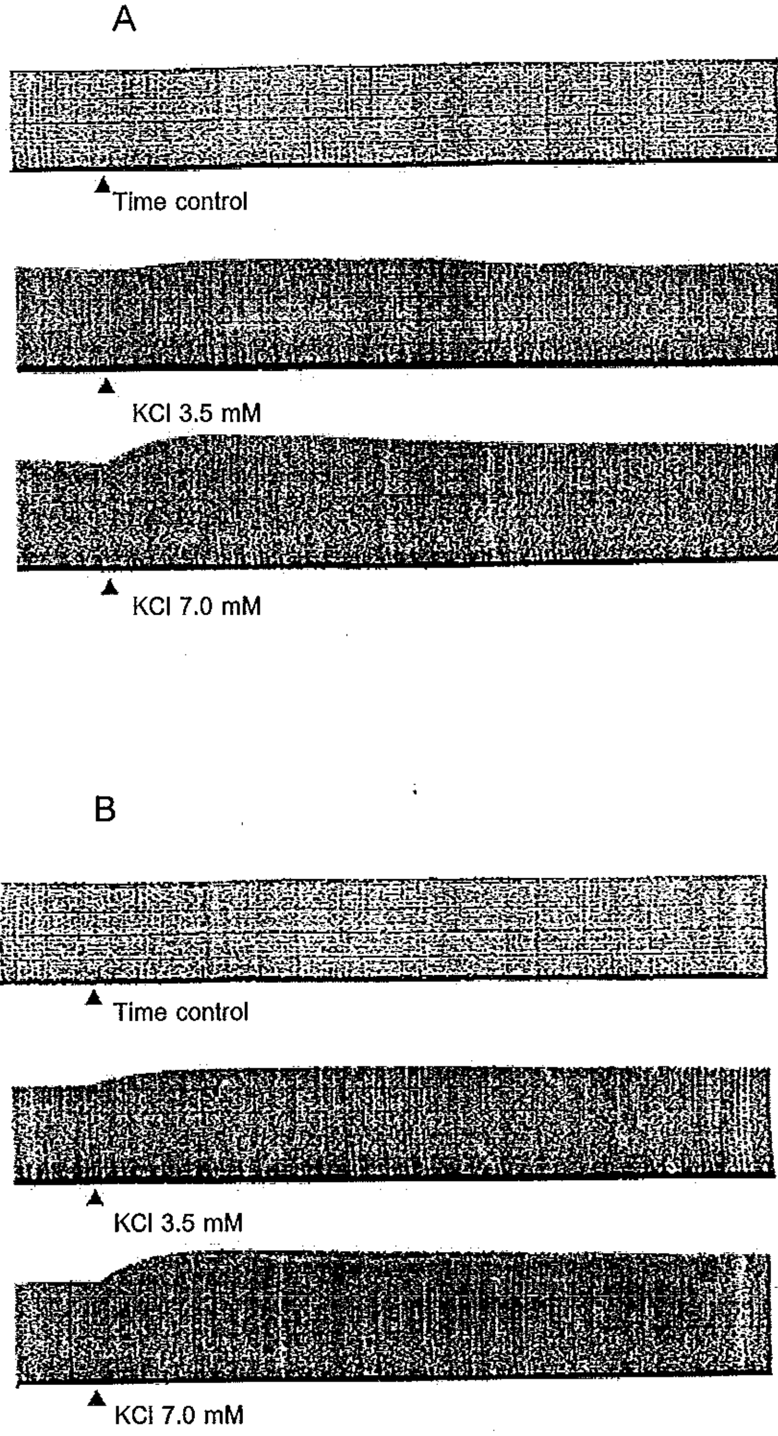


Figure 36. The representative tracings of KCl (3.5 and 7.0 mM) on neurally-evoked twitch (A) and Directly-evoked twitch (B).

Table 11. The time-action relationships of KCl (3.5 and 7.0 mM) on neurally- and directly-evoked twitch.

The data were presented as mean ± SE of 6 observations.

Time (min)	Twitch tension (% Control)			
	Neurally-evoked twitch		Directly-evoked twitch	
	KCl 3.5 mM	KCl 7.0 mM	KCl 3.5 mM	KCl 7.0 mM
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
10	112.02 ± 1.39	125.35 ± 1.94	113.43 ± 1.59	121.77 ± 2.33
20	111.73 ± 1.12	123.39 ± 2.04	111.83 ± 2.46	119.66 ± 3.28
30	109.49 ± 1.20	118.84 ± 2.98	110.83 ± 3.09	115.76 ± 3.73
40	107.94 ± 1.69	116.66 ± 3.31	109.79 ± 3.92	113.04 ± 4.03
50	106.58 ± 1.60	113.26 ± 3.59	108.60 ± 4.00	110.97 ± 4.40
60	106.08 ± 1.53	111.31 ± 3.52	107.96 ± 4.60	109.24 ± 3.93

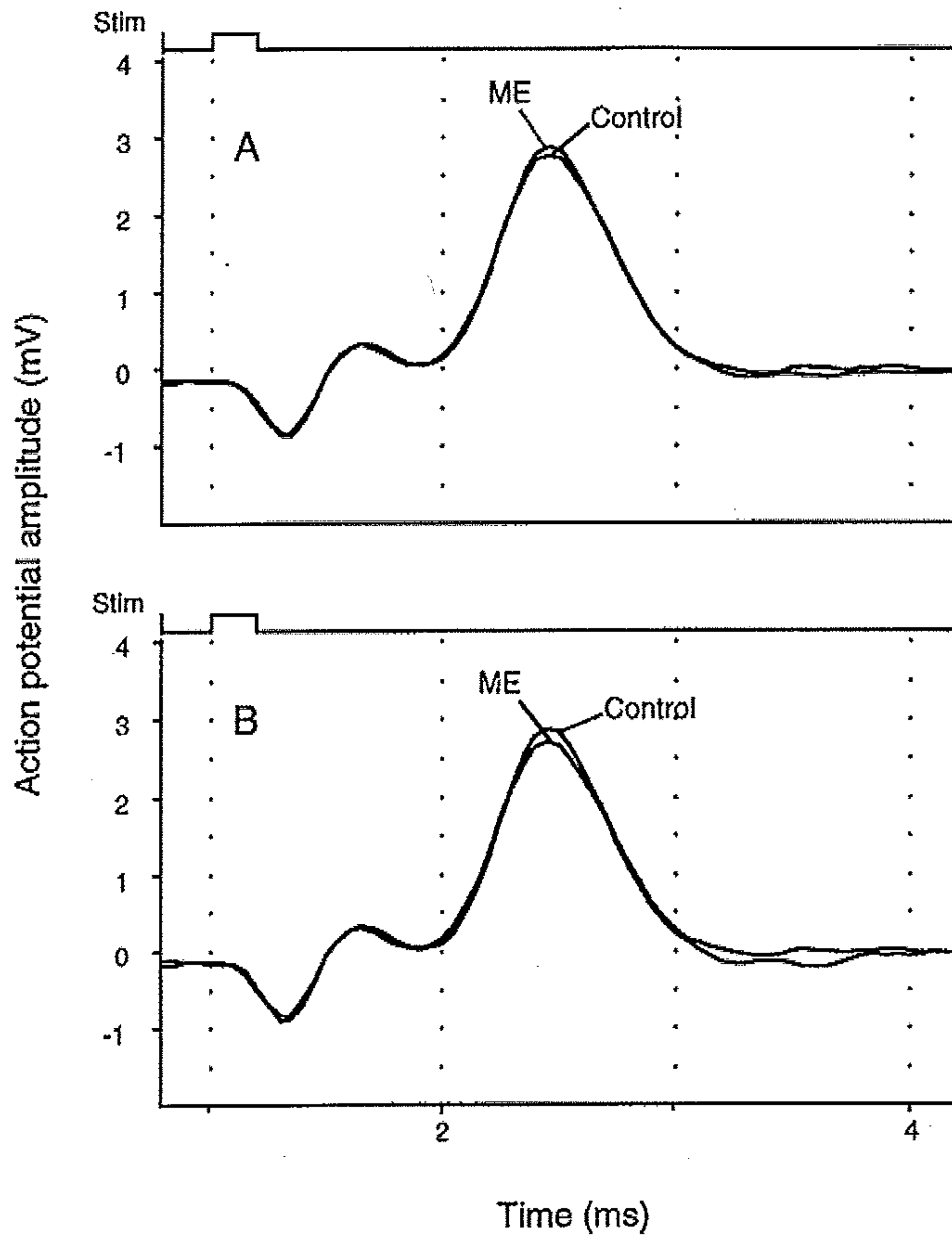


Figure 37. The action potential amplitudes in the presence of ME (4.0 mg/ml) at 10 minutes (A) and 30 minutes (B); n = 6.

Table 12. The effect of ME (4.0 mg/ml) on the action potential amplitude in the isolated sciatic nerve preparation. The data were presented as mean ± SE of 6 observations. There was not significant difference from control ($P > 0.05$).

Time (min.)	Action potential amplitude (% of control)	
	Control	ME 4.0 mg/ml
0	100.00	100.00
10	96.31 ±1.23	98.57 ± 2.96
20	97.40 ± 3.15	95.45 ± 3.73
30	95.11 ± 3.63	93.18 ± 4.91

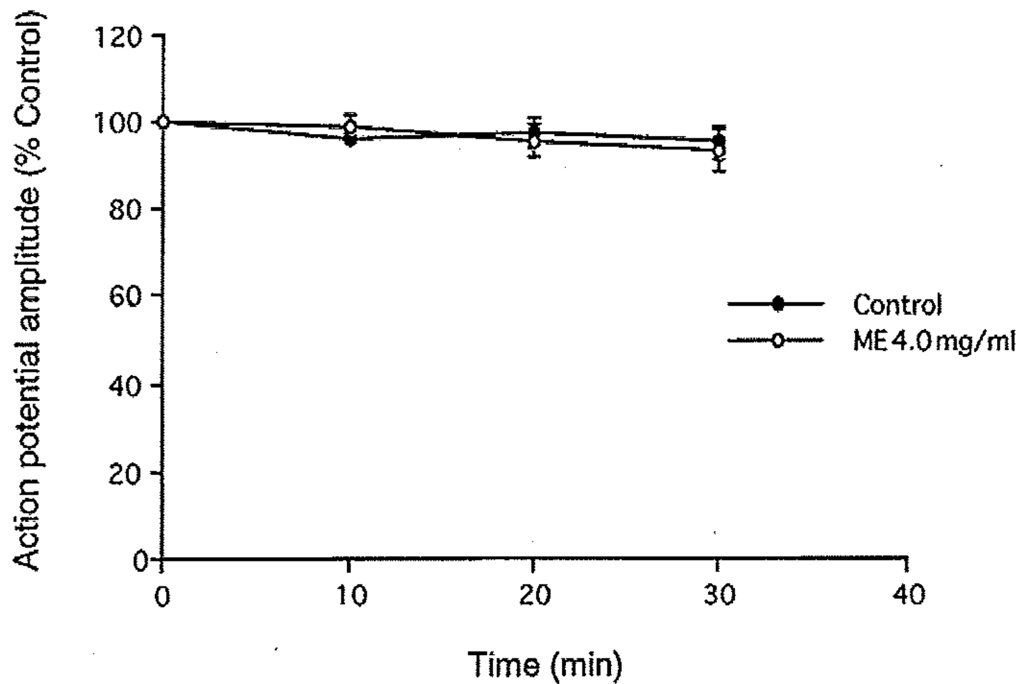


Figure 38. The effects of ME (4.0 mg/ml) on the action potential amplitude in isolated sciatic nerve preparation. Each point is mean ± SE of 6 observations.

action potential in control group was slightly decreased about 4.98 ± 3.63 % at 30 minutes. In the presence of 4.0 mg/ml of ME, the action potential amplitude was slightly decreased about 6.82 ± 4.91 % at 30 minutes which was not significant difference from control ($P > 0.05$) at every time measured as shown in Table 12 and Figure 37 and 38.

Section 2 Interaction of ME and Drugs or Agents affecting on neuromuscular junction

Part A The Synergistic Effect Studies.

These experiments were set up in order to observe the effects of ME in the presence of the standard neuromuscular blocking agents, i.e., dTC or SCh, after adding dTC or SCh for 3 minutes. The effects were compared to those of the standard neuromuscular blocking agents alone.

1. The effect of ME in the presence of dTC

When ME at final concentration of 1.6 mg/ml was added into the organ bath containing 1.0 μM of dTC, the percent twitch depression produced by ME was significantly less than control ($P < 0.05$). In the other hand, when ME at concentration of 3.2 mg/ml was added into the organ bath containing 1.0 μM of dTC, the percent twitch depression was significantly greater than control ($P < 0.05$) as

Table 13. Comparison of the twitch depression produced by ME (1.6 or 3.2 mg/ml) in the presence of dTC (1.0 μM).

No. of Observation	% Twitch depression produced by		
	dTC (1.0 μM)	[a]ME 1.6 mg/ml after dTC	[b]ME 3.2 mg/ml after dTC
1	61.76	54.05	88.46
2	47.83	18.78	100.00
3	56.00	38.10	80.95
4	37.50	36.36	80.95
5	55.56	46.96	93.75
6	48.31	41.33	83.87
7	53.04	51.55	84.87
8	56.76	54.70	84.21
Mean ± SE	52.10 ± 2.64	42.70 ± 4.22 *	87.13 ± 2.36*

Note: 1. Superscripts *"a"* and *"b"* define that ME was added after dTC 3 minutes.

2. Twitch depression measured at 30 minutes after adding dTC.

3. Asterisk (*) Indicates significant difference from control ($P < 0.05$).

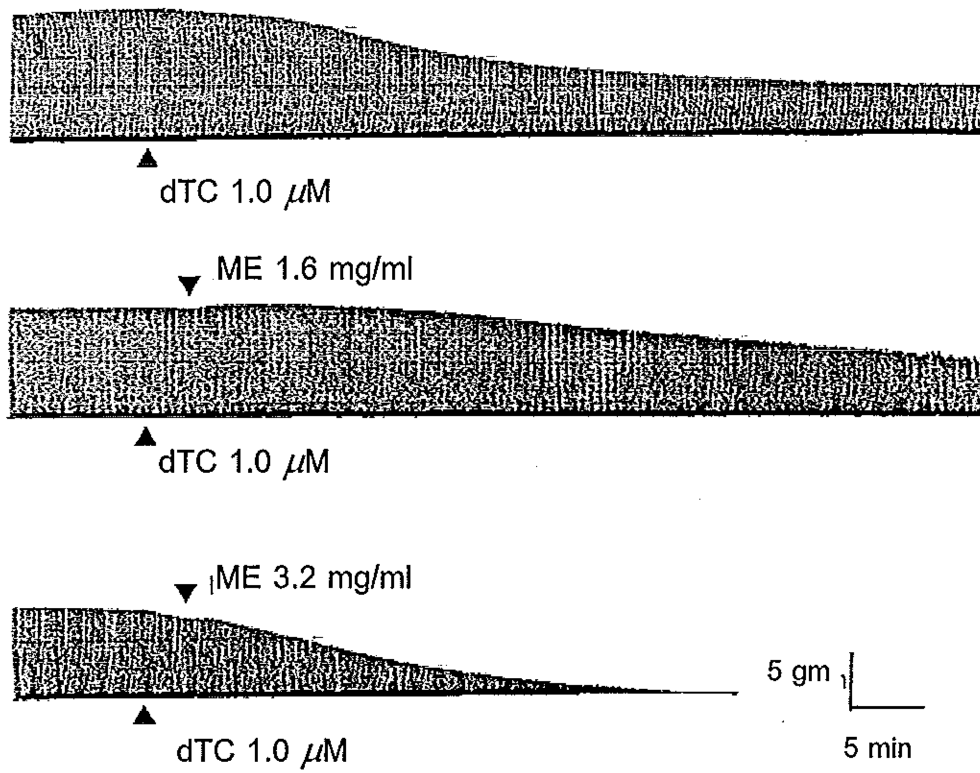


Figure 39. The effects of ME (1.6 and 3.2 mg/ml) in the presence of dTC (1.0 μM) on neurally-evoked twitch.

indicated in Table 13. The representative tracings was shown in Figure 39.

2. The effect of ME in the presence of SCh

When ME at final concentrations of 1.6 or 3.2 mg/ml were added to the organ bath containing 10 μM of SCh. It was found that the percent twitch depression produced by ME was significantly greater than control ($P < 0.05$) in both concentration as indicated in Table 14. The representative tracings were shown in Figure 40.

Part B The antagonistic effect studies.

These experiments were set up in order to observe the effects of ME in the presence of the standard neuromuscular blocking agents i.e., dTC or SCh, after adding dTC or SCh for 30 minutes to induced 50% neuromuscular blockade. The effects were compared to those of the standard neuromuscular blocking agents alone.

1. The effect of ME after dTC

When ME at final concentrations of 1.6 or 3.2 mg/ml were added into the organ bath containing 1 μM dTC, it was found that the twitch depression could be antagonized in a small amount and followed by the percent twitch depression which was significantly greater than each control ($P < 0.05$). Furthermore, ME at concentration

Table 14. Comparison of the twitch depression produced by ME (1.6 or 3.2 mg/ml) in the presence of SCh (10 μM).

No. of Observation	% Twitch depression produced by		
	SCh (10 μM)	[a]ME 1.6 mg/ml after SCh	[b]ME 3.2 mg/ml after SCh
1	22.22	81.48	100.00
2	16.36	89.47	100.00
3	19.15	90.53	100.00
4	14.29	97.50	100.00
5	23.81	95.20	100.00
6	21.37	66.67	100.00
7	25.93	85.94	100.00
8	19.78	92.50	100.00
Mean ± SE	20.36 ± 1.35	87.41 ± 3.46*	100.00 ± 0.00*

Note: 1. Superscripts *"a"* and *"b"* define that ME was added after SCh 3 minutes.

2. Twitch depression was measured at 20 minutes after adding SCh.

3. Asterisk (*) Indicates significant difference from control ($P < 0.05$).

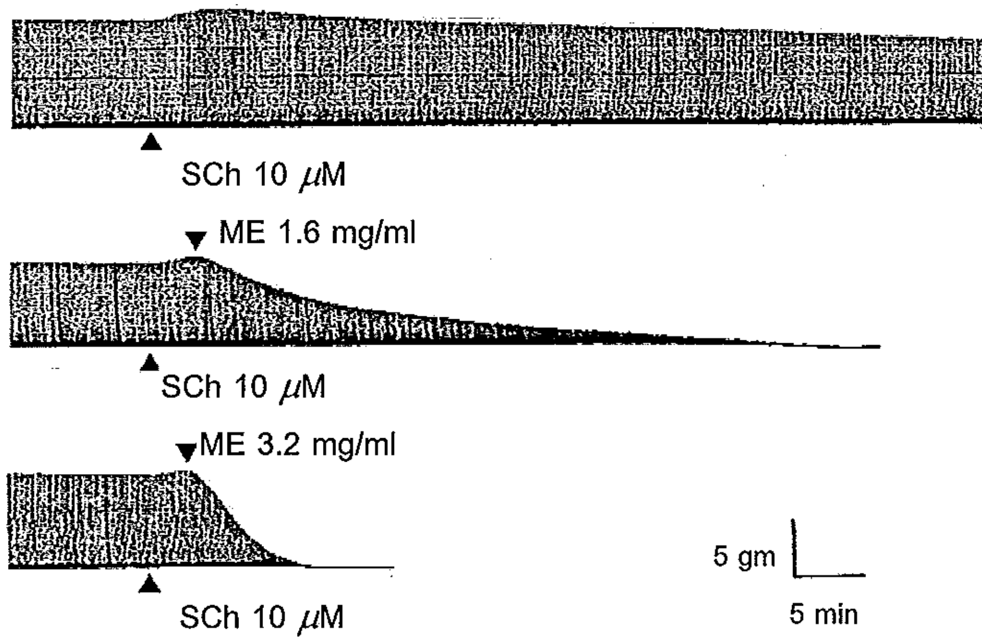


Figure 40. The effects of ME (1.6 and 3.2 mg/ml) in the presence of SCh (10 μM) on neurally-evoked twitch.

Table 15. Comparison of the twitch depression produced by ME (1.6, 3.2 mg/ml) in the presence of dTC (1.0 μM) induced 50 % neuromuscular blockade.

No. of observation	% Twitch depression produced by			
	dTC[a] (1.0 μM)	ME 1.6 mg/ml after dTC[c]	dTC[b] (1.0 μM)	ME 3.2 mg/ml after dTC[d]
1	44.44	72.22	52.28	90.99
2	52.63	68.42	61.54	97.41
3	45.68	75.31	43.48	100.00
4	50.00	67.67	54.29	100.00
5	43.25	56.25	63.16	100.00
6	56.41	84.62	55.00	100.00
7	47.06	67.65	52.94	100.00
Mean ±SE	48.50 ±1.80	70.31 * ±3.27	54.67 ±2.46	98.33 * ±1.29

Note: 1. Superscripts *"a"* and *"b"* define as twitch tension was measured at 30 minutes.

2. Superscripts *"c"* and *"d"* define as twitch tension was measured at 60 minutes.

3. Asterisk (*) indicates significant difference from control ($P < 0.05$)

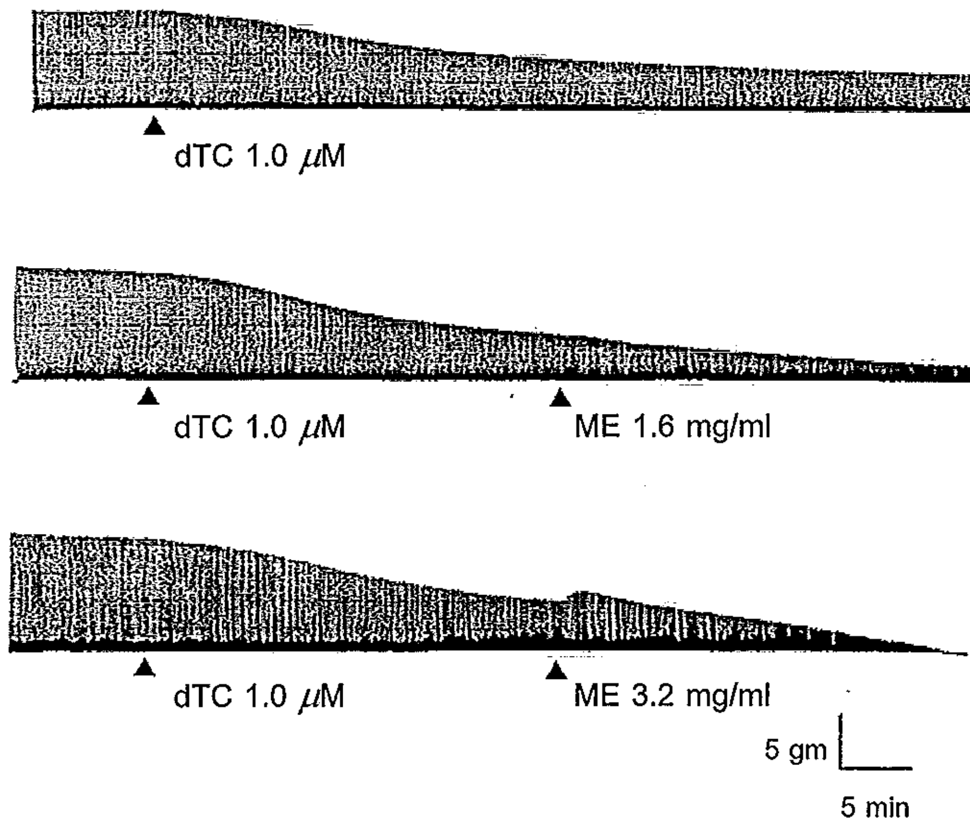


Figure 41. The effects of ME (1.6 and 3.2 mg/ml) on dTC (1.0 μM) induced 50 % neuromuscular blockade.

of 3.2 mg/ml could produce twitch depression significantly greater than ME at concentration of 1.6 mg/ml as indicated in Table 15. The representative tracings were shown in Figure 41.

2. The effect of ME after SCh

When ME at final concentrations of 1.6 or 3.2 mg/ml were added into the organ bath containing 15 μM of SCh, it was found that the percent twitch depression was significantly greater than each control ($P < 0.05$). Furthermore, both concentrations of ME could produce a rapid neuromuscular block as indicated in Table 16. The representative tracings were shown in Figure 42.

3. The antagonistic effects of neostigmine on 50% neuromuscular block induced by dTC or ME.

These experiments were set up in order to observe the effects of the standard antagonistic agents, neostigmine (NS) or tetraethylammonium (TEA) on 50% dTC induced neuromuscular block compared to that on 50% ME induced neuromuscular blockade.

In this investigation, the neuromuscular transmission was blocked for 50% by dTC (1 μM) or ME (4.0 mg/ml) which was taken about 30 minutes. When Neostigmine (5 μM) was added at that point, it was found that neostigmine could antagonize those effects

Table 16. Comparison of the twitch depression produced by ME (1.6, 3.2 mg/ml) in the presence of SCh (15 μM) induced 50 % neuromuscular blockade.

No. of observation	% Twitch depression produced by			
	SCh[a] (15 μM)	ME 1.6 mg/ml after SCh[c]	SCh[b] (15 μM)	ME 3.2 mg/ml after SCh[d]
1	52.78	100.00	50.00	100.00
2	60.61	100.00	68.00	100.00
3	46.67	100.00	47.06	100.00
4	50.00	100.00	35.29	100.00
5	42.11	100.00	47.46	100.00
6	55.77	100.00	54.45	100.00
Mean ± SE	51.32 ± 2.69	100.00 * ± 0.00	50.38 ± 4.38	100.00 * ± 0.00

Note: 1. Superscripts *"a"* and *"b"* define as twitch tension was measured at 20 minutes.

2. Superscripts *"c"* and *"d"* define as twitch tension was measured at 40 minutes.

3. Asterisk (*) indicates significant difference from control ($P < 0.05$)

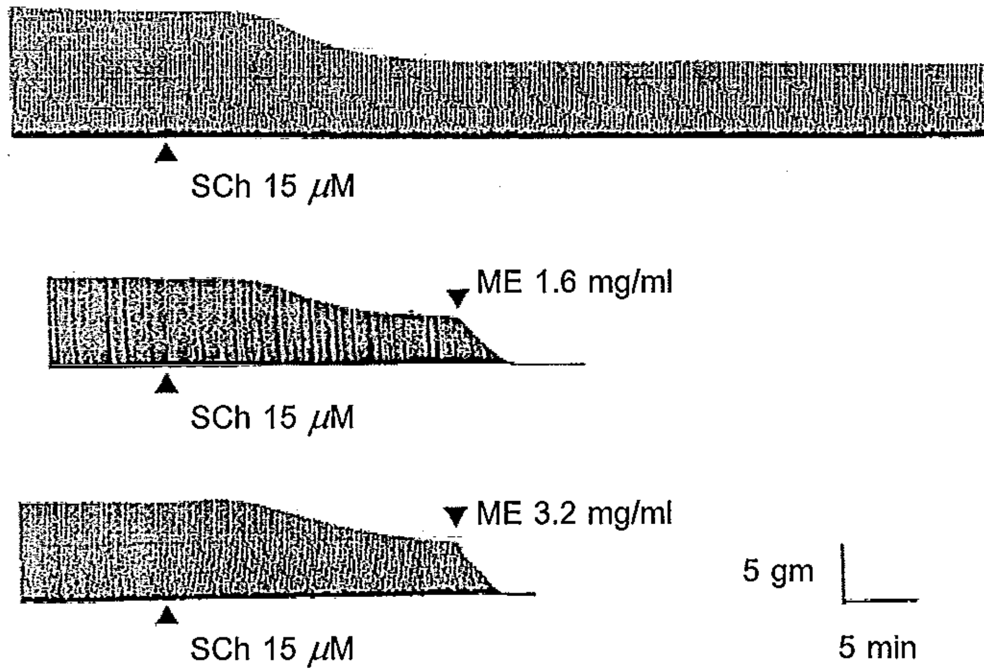


Figure 42. The effects of ME (1.6 and 3.2 mg/ml) in SCh (15 μM) induced 50% neuromuscular blockade.

Table 17. Effect of neostigmine (5 μM) on 50% neuromuscular blockade induced by dTC (1 μM) or ME (4.0 mg/ml).

No. of observation	[a]Neuromuscular block induced by dTC	[b]% Antagonized by neostigmine	[a]Neuromuscular block induced by ME	[b]% Antagonized by neostigmine
1	51.43	92.22	45.45	0.00
2	64.71	89.09	56.25	0.00
3	73.33	90.91	44.44	0.00
4	48.48	100.00	52.38	0.00
5	52.94	88.89	47.37	0.00
6	56.41	95.45	45.00	0.00
Mean ± SE	57.88 ± 3.84	92.24 ±1.82	48.48 ±1.95	0.00 ± 0.00*

Note: 1. Superscript *"a"* defines as neuromuscular blockade was measured at 30 minutes after administration.

2. Superscript *"b"* defines as maximum antagonized.

3. Asterisk (*) indicates significant difference from control ($P < 0.05$)

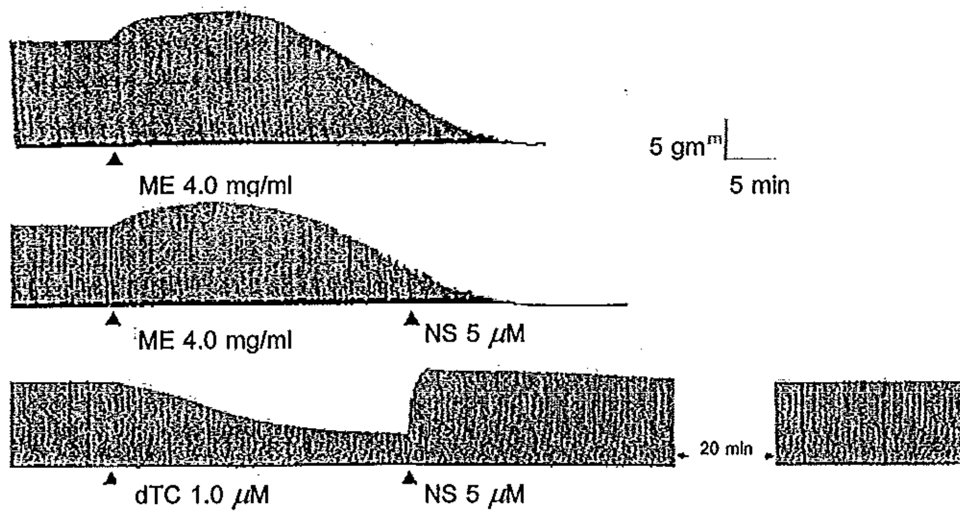


Figure 43. The effects of neostigmine (NS, 5 μM) on 50% neuromuscular blockade produced by d-tubocurarine (dTC, 1 μM) and ME (4.0 mg/ml) on neurally-evoked twitch.

produced by dTC but not ME as shown in Table 17 and Figure 45. The representative tracings were shown in Figure 43.

4. The antagonistic effects of tetraethylammonium (TEA) on 50% neuromuscular block induced by dTC or ME.

In the same manner to previous investigation, the neuromuscular transmission was blocked for 50% by dTC (2 μM) or ME (4.0 mg/ml) which was taken about 30 minutes. It was found that TEA (1mM) could antagonize the 50% neuromuscular block induced by dTC but the antagonistic effect was transient then followed by a gradual twitch depression. In the same manner, TEA could transiently antagonize the twitch depression produced by ME and then followed by progressive complete twitch depression as shown in Table 18 and Figure 45. The representative tracings were shown in Figure 44.

Part C Effects of ME on acetylcholine contraction.

It was proposed that acetylcholine (ACh) contraction was due to the interaction of acetylcholine with nicotinic receptor on motor endplate (Thesleft, 1959). A sensitive method to determine the postsynaptic action of drug is an investigation of the drug action on ACh contraction (Kuffler, 1943). Sufficient dose of ACh in non stimulated nerve-muscle preparation, produce endplate depolarization, leading to muscle contraction (Koelle, 1975).

In this investigation, the freshly rat phrenic nerve-hemidiaphragm preparation was performed for recording muscle contraction without electrical stimulation. When ACh at the dose of 30 mM was administrated, it was found that ACh was capable to produce a muscle contraction as shown in Figure 46 A. When dTC (2 μM) or ME (4.0 mg/ml) was given 5 minutes before ACh (30 mM), it was found that both dTC or ME could completely inhibit ACh contraction as shown in Figure 46 B and C, respectively.

Table 18. Effect of TEA (1 mM) on 50% neuromuscular blockade induced by dTC (1 μM) or ME (4.0 mg/ml).

No. of observation	[a]Neuromuscular block induced by dTC	[b]% Antagonized by TEA	[a]Neuromuscular block induced by ME	[b]% Antagonized by TEA
1	56.10	86.96	50.00	54.55
2	53.66	93.64	57.89	27.27
3	56.52	96.15	57.89	18.18
4	62.79	96.30	54.55	16.47
5	45.65	100.00	50.00	30.00
6	55.00	109.09	46.15	133.33
Mean ± SE	54.95 ± 2.26	97.02 ± 2.99	52.75 ± 1.96	46.67 ± 18.20*

Note: 1. Superscript *"a"* defines as neuromuscular blockade was measured at 30 minutes after administration.

2. Superscript *"b"* defines as maximum antagonized.

3. Asterisk (*) indicates significant difference from control ($P < 0.05$)

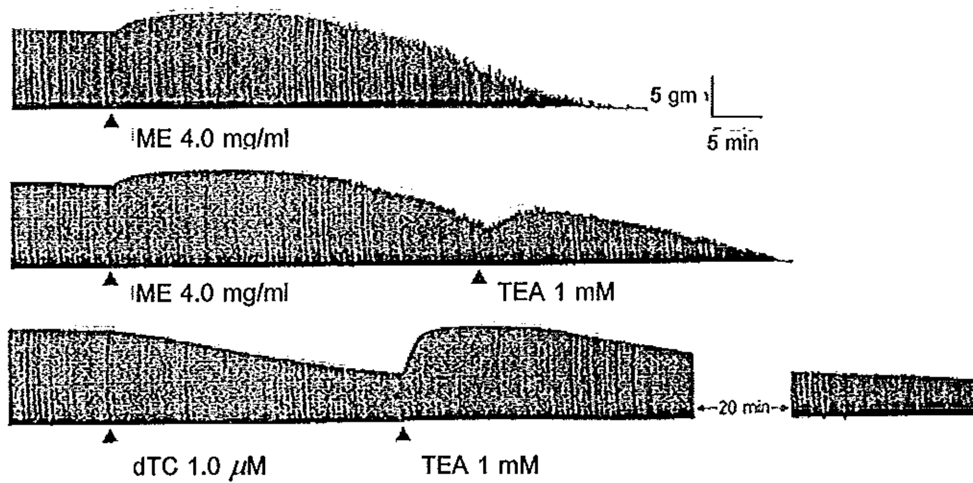


Figure 44. The effects of tetraethylamonium (TEA, 1 mM) on 50% neuromuscular blockade produced by d-tubocurarine (dTC, 1 μM) and ME (4.0 mg/ml) on neurally-evoked twitch.

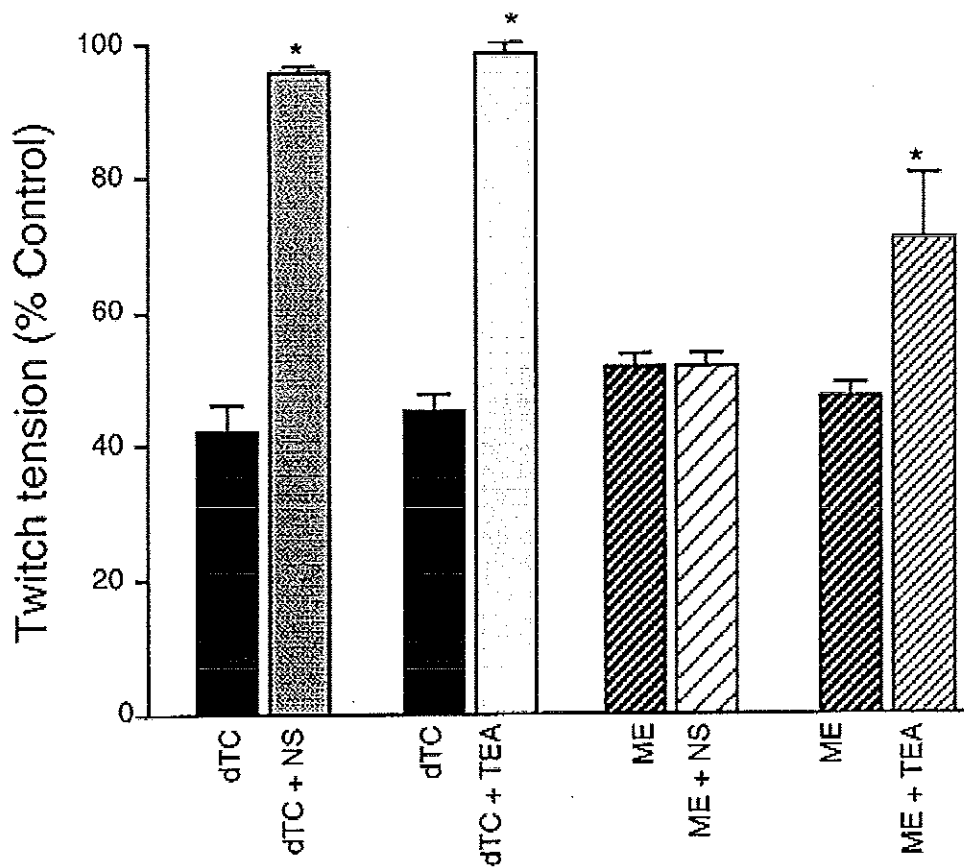


Figure 45. Comparison of the effects of neostigmine (NS, 5 λM) and tetraethylamonium (TEA, 1 mM) on 50% neuromuscular blockade produced by d-tubocurarine (dTC, 1 μM) and ME (4.0 mg/ml) on neurally-evoked twitch. Each column represents the mean in twitch tension (% control) of 6 experiments while bars indicate the SE. Asterisk (*) indicates significant difference from control ($P<0.05$)

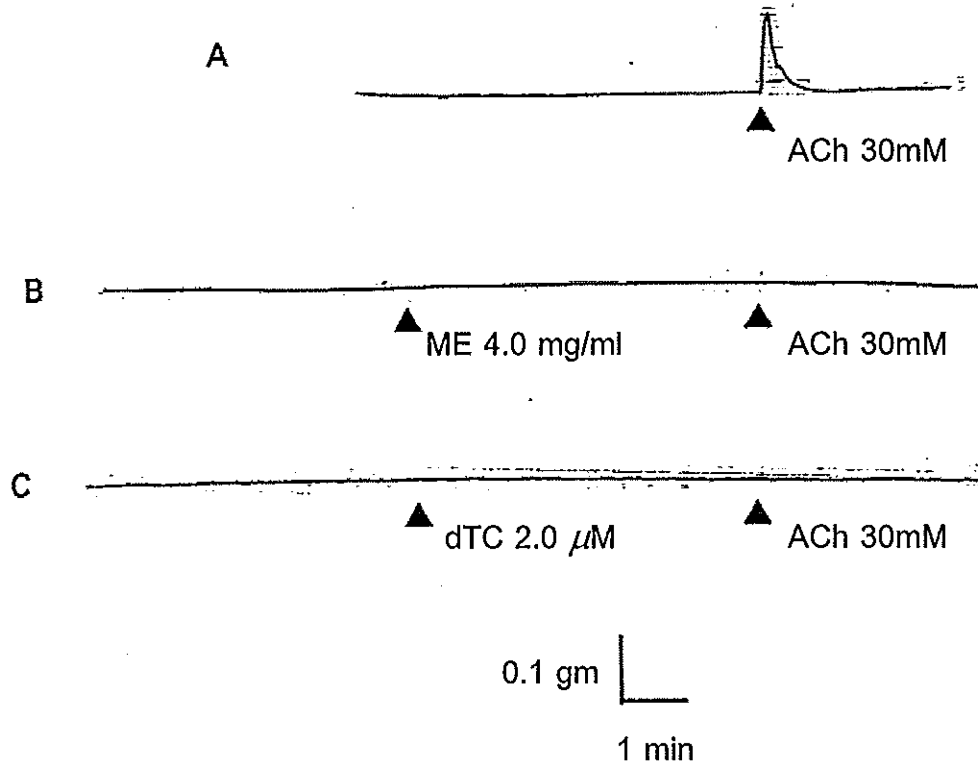


Figure 46. The effects of ME on acetylcholine contraction

A. Control, acetylcholine contraction produced by ACh (30 mM).

B and C: ME (4.0 mg/ml) and dTC (2 μM) suppressed acetylcholine contraction, respectively.

CHAPTER 5

DISCUSSION

In the present investigation, the crude methanol extract from *Piper sarmentosum* Roxb. (ME) produced dual effects on contractile responses, twitch potentiation followed by twitch depression, in isolated rat phrenic nerve-hemidiaphragm preparation both neurally- and directly-evoked twitch without depressive effect on nerve action potential. The ME at low concentrations could significantly reduce the depressive effect on the preparation in the presence of non-depolarizing neuromuscular blocking agent, d-tubocurarine (dTC), but at higher concentrations, ME significantly synergized depressive effect. The ME could only produce synergistic effect on the preparation in the presence of the depolarizing neuromuscular blocking agent, succinylcholine (SCh). The twitch depression induced by ME could transiently and partialy antagonized by tetraethyl-ammonium (TEA) but not by neostigmine (NS). Both ME and dTC completely suppressed the effect of ACh contraction.

According to these results, the twitch depression produced by ME on neurally-evoked twitch was compared to that of directly-evoked twitch. At 30 minutes after adding ME, it was found that ME

(3.2-4.8 mg/ml) in neurally-evoked twitch produced twitch depression significantly greater than that in directly evoked twitch ($P < 0.05$). In addition, ME did not affect on nerve action potential. It would be proposed that the twitch depression of ME was mainly due to the interference of neuromuscular transmission. However, the twitch depressive effect of ME might be one of the consideration of the direct effect on muscle, while the twitch potentiation of the neurally-evoked twitch produced by low concentrations of ME was no significant difference from that of directly-evoked twitch. The direct effect of ME on muscle should be further studied in details.

Since the ME is a crude methanol extract, it is composed of many chemicals and ions. Those ions might have an effect on neuromuscular transmission or muscle. The ions composition in ME were determined by ICPaes method (Varma, 1991) and it was found that K^+ is the major ion in ME. This ion is one of the ions containing in Krebs' solution and it is also present in ME. Therefore, the twitch potentiation of ME was studied in comparison to the effect of K^+ in this preparation. K^+ at concentrations of 3.5 and 7.0 μM which equal to concentration of K^+ in ME at concentrations of 1.6 and 3.2 mg/ml, respectively were used for investigation in both neurally- and directly-evoked twitch. It was found that both concentrations of K^+ produced only twitch potentiation. It could be postulated that the twitch potentiation of ME probably induced by K^+. This ion influenced on

neuromuscular transmission or muscle by producing a direct depolarization on the muscle membrane including motor endplates (Kuffler, 1945). During the process of depolarization, the excess of K^+ efflux would be an important ion that tended to produce excitable membrane-endplate (Hubbard, 1973). Thus the twitch potentiation of ME possibly was due to the potentiation action of K^+ containing in the ME.

When acetylcholine or another agonist is applied to the motor endplates for prolonged periods, the endplate membrane does not remain at the level of depolarization produced initially. Instead the response declines to reach a steady state that is virtually close to its resting level despite the continued application of the agonist. Although the membrane potential may recover to normal, neuromuscular transmission remains blocked throughout the drug application. The declining response is said to be caused by conformational changes in receptor or receptor desensitization (Haspel, *et al.,* 1993). Thus the blockade of neuromuscular junction of ME seemed to be considered as a depolarized neuromuscular blockade.

The depolarizing agents, such as succinylcholine and decamethonium, act by their initial action to depolarize the membrane by opening channels in the same manner as ACh. However, since they persist at neuromuscular junction, the depolarization is longer lasting resulted in a brief period of repetitive excitation, which may be

manifested by transient muscle fasciculation and potentiation of the maximum twitch. This phase followed by block of neuromuscular transmission and flaccid paralysis. This block is potentiated by anticholinesterase agents. However, following the onset of blockade, their is poorly sustained response to tetanic stimulation of motor nerve, intensification block by dTC, and usual reversal by anticholinesterase agents (Taylor, 1991).

Assume that the ME act on neuromuscular junction with same manner as depolarizing agents, its effect in this investigation was according to the description of Taylor (1991) as mentioned above. It was shown in this investigation that the neuromuscular depression of ME was not only preceded by a slight increase in twitch tension, but also found that low concentrations of ME could reduce the depressive effect of dTC. Furthermore, the ME could produce synergistic effect on preparation in the presence of SCh.

It is interesting that while low concentrations of ME could reduce the depressive effect but higher concentrations exhibited the enhancement on depressive effect of dTC. Perhaps higher concentrations of ME might act on neuromuscular junction like a non-depolarizing agent.

The non-depolarizing neuromuscular blocking agents bind to and compete with ACh for the recognition sites (alpha subunits) of the nicotinic receptors but are devoided of the property called "efficacy"

or “intrinsic activity”. That is to say, they are incapable of producing the conformation change in the receptor protein that constitutes opening of the ion channel. Hence, they act merely to impede the access of ACh to the receptors. The dTC is classical example of a reversible competitive antagonist, and certainly its effect can be overcome by increasing the concentration of ACh, e.g. with neostigmine. In this investigation, the ME at higher concentration exhibited the enhancement on depressive effect of dTC, therefore, it seemed to be increasing the action of non-depolarizing agents. In addition, the depressive effect of ME could not be antagonized by an anticholinesterase agent, neostigmine while tetraethylammonium could partially transient antagonize.

Braga and coworkers (1993) described that neostigmine is a powerful anticholinesterase agent. The action of neostigmine was clearly to exert on the transmission mechanism, because it had no effect on muscle contraction induced by direct muscle stimulation. Although inhibition of acetylcholinesterase is the main mechanism of its action, it also exerts an additional direct action on motor nerve endings to produce some degree of block of the delayed rectifier K^+ channels causing the enhancement of transmitter release, its ability to interact directly with postjunctional acetylcholine receptors requires very large concentration. In another experiment, Braga and coworkers (1992) have reported that nicotinic agonists may directly enhance the

inward Ca^{+} current at nerve ending to cause the enhance release of ACh which causes in increasing in twitch tension. Therefore, it seemed likely that accumulating of ACh in the presence of anticholinesterase agent would also produce this action.

Kensler (1950) reported that tetraethylammonium (TEA) is a quaternary ammonium compound, producing the effect on neuromuscular transmission by augmentation the response of skeletal muscle to nerve stimulation. Stovner (1957), suggested that TEA increase ACh liberation from motor nerve ending in cat. TEA is an effective agent in blocking calcium-activated K^{+} channels that are present at the motor nerve terminal (Penner and Dreyer, 1986). Unfortunately, TEA also has a pronounced inhibitory action on the nicotinic ion channel in the same concentration range that it produces enhancement of transmitter release (Adler, *et al.*, 1979).

Anderson and colleagues (1988) have described that the Ca^{2+} that enters not only activates the ACh release mechanism, but also acts to open a population of calcium-activated K^{+} channels. Hence, there is a secondary efflux of K^{+} ions (I_{KCa}) which presumably accelerates the repolarization of the membrane and thereby closes the Ca^{2+} channels. It may be that the secondary I_{KCa} acts as a breaking current on Ca^{2+} entry to prevent the toxic consequence of excess intraterminal Ca^{2+}.

When the I_{KCa} was blocked by TEA causing to increase of Ca^{2+} that caused ACh liberation from motor nerve ending. In this experiment TEA could transient partially antagonize the depressive effect produced by ME. This point could support that ME at higher concentrations might produce a higher degree of blockade by releasing of ACh from the nerve terminal.

Another consideration of the nature of neuromuscular blockade produced by ME was carried out on ACh contraction which is a sensitive method to determine the action of drugs on postsynaptic by ACh induced muscle contraction without electrical stimulation (Thesleft, 1959). This phenomenon of ACh contraction was proposed to be the interaction of ACh with nicotinic receptor at the motor endplates (Dale, *et al.*, 1936), causing to produce a depolarization on motor endplate called endplate potential (EPP). A endplate potential induces a muscle action potential. If it interact with a sufficient concentration of ACh, the muscle will contract by itself.

It was found that both dTC and ME could suppress amplitude of ACh contraction. The dTC produce neuromuscular blockade by binding to and competing with ACh for the recognition sites of the nicotinic receptors but they are incapable of producing the conformation change in the receptor protein that constitutes opening of the ion channel. This action of dTC is causing to decrease in EPP to below threshold level, then no contraction occurred. The effect of ME

on ACh contraction was similar to that of dTC. Therefore, the one possibility to propose the site of neuromuscular blockade of ME was postsynaptic membrane (motor endplate). Assuming that ME acted on the postsynaptic membrane like dTC, it would propose that ME competed with ACh for nicotinic receptors, but there is no intrinsic activity, so ACh contraction was abolished. In this study, the effect of ME on EPP was not investigated.

Another possibility of neuromuscular blockade produced by ME was a decrease in the sensitivity of the endplate to ACh. The nicotinic receptors are less sensitive to react with ACh or the nature of the receptors was altered or conversed by ME action. It was concluded that ME might have a postsynaptic action by competition with ACh for nicotinic receptor or decreasing in the sensitivity of the endplate to ACh, or alteration of nicotinic receptors.

From the present study, it would be concluded as follow (Figure 47).

1. ME produced a dual effect on contractile responses, twitch potentiation and followed by twitch depression, in the isolated rat phrenic nerve-hemidiaphragm preparation both neurally- and directly-evoked twitch without depressive effect on nerve action potential conduction.

2. The twitch potentiation effect of ME (low concentrations) was mainly related to its direct action on muscle membrane and this

effect was possibly due to the potentiation action of K^+ containing in the ME.

3. The twitch depression was predominantly due to the interference of neuromuscular transmission. It would be proposed that the twitch depression of ME was mainly due to presynaptic action by decreasing in transmitter release from motor nerve terminal.

The postsynaptic site would also consider being the site of neuromuscular blockade, in which ME might act as a competitive neuromuscular blocking drugs and/or proposed to be a decreasing the endplate sensitivity to ACh.

4. The twitch depressive effect of ME might be due to the direct effect on muscle.

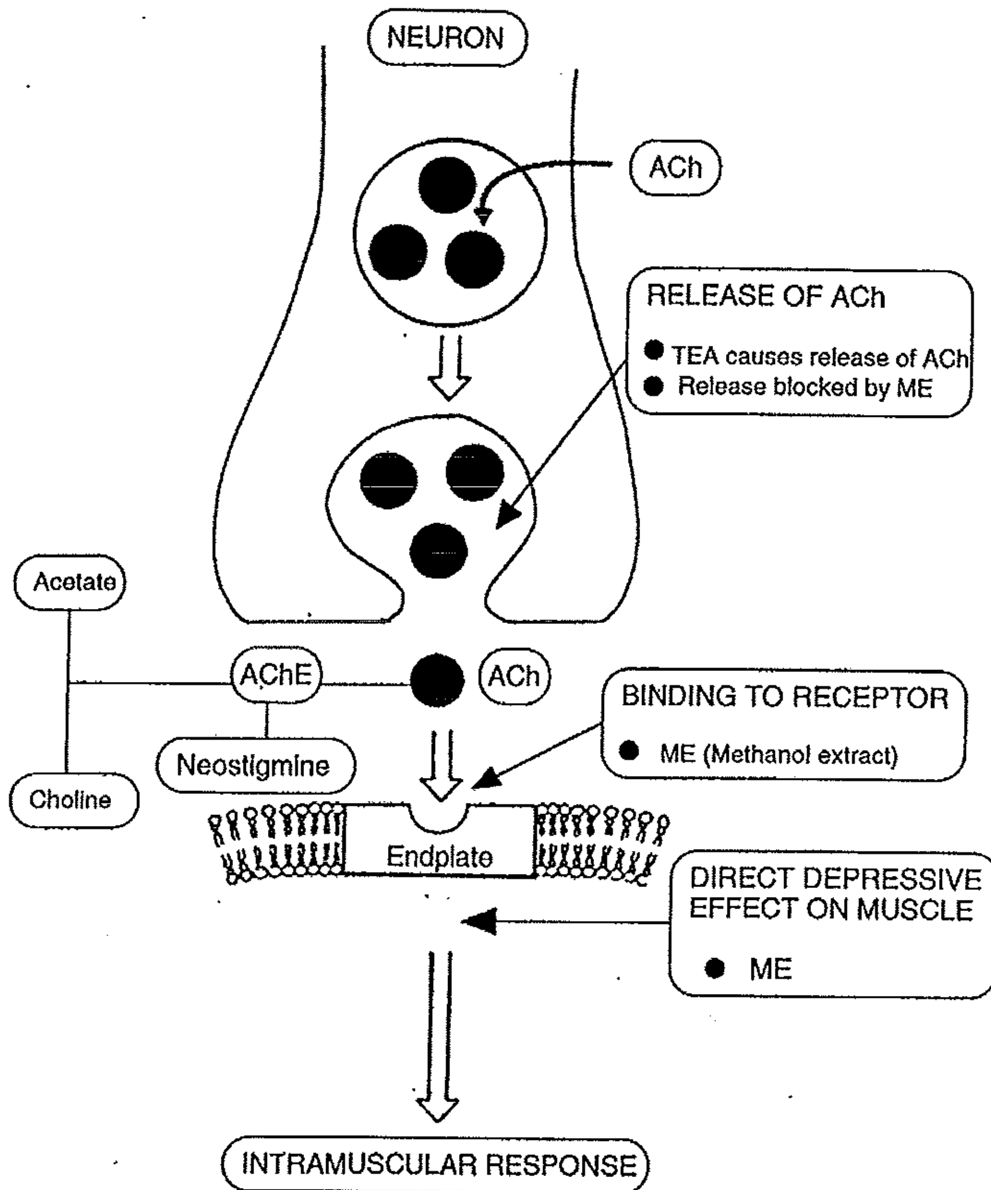


Figure 47. Proposed sites and mechanism of neuromuscular blockade caused by ME at the neuromuscular junction.

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