

Studies on Neuromuscular Blockade by Boldine in the Mouse Phrenic Nerve-Diaphragm

Jaw-Jou Kang¹, Yu-Wen Cheng and Wen-Mei Fu²

¹Institute of Toxicology and ²Department of Pharmacology, College of Medicine, National Taiwan University, No. 1 Jen-Ai Road, Section 1, Taipei, Taiwan

Received August 11, 1997 Accepted November 19, 1997

ABSTRACT—The effects of boldine [(*S*)-2,9-dihydroxyl-1,10-dimethoxy-aporphine], a major alkaloid in the leaves and bark of Boldo (*Peumus boldus* Mol.), on neuromuscular transmission were studied using a muscle phrenic-nerve diaphragm preparation. Boldine at concentrations lower than 200 μ M preferentially inhibited, after an initial period of twitch augmentation, the nerve-evoked twitches of the mouse diaphragm and left the muscle-evoked twitches unaffected. The twitch inhibition could be restored by neostigmine or washout with Krebs solution. The twitches evoked indirectly and directly were both augmented initially, suggesting that the twitch augmentation induced by boldine was myogenic. Boldine inhibited the acetylcholine-induced contraction of denervated diaphragm dose-dependently with an IC_{50} value of 13.5 μ M. At 50 μ M, boldine specifically inhibited the amplitude of the miniature end plate potential. In addition, boldine was similar to *d*-tubocurarine in its action to reverse the neuromuscular blocking action of α -bungarotoxin. These results showed that the neuromuscular blockade by boldine on isolated mouse phrenic-nerve diaphragm might be due to its direct interaction with the postsynaptic nicotinic acetylcholine receptor.

Keywords: Boldine, *Peumus boldus* Mol., Neuromuscular blockade, Acetylcholine channel

Boldine [(*S*)-2,9-dihydroxyl-1,10-dimethoxy-aporphine], a papaverine related aporphine alkaloid, is a major alkaloid found in the leaves and bark of Boldo (*Peumus boldus* Mol.) (1) that has been shown to exert several distinct pharmacological actions (2, 3). It has been shown to prevent autooxidation of the brain homogenate and lipid peroxidation of red cell membrane (4), have anti-inflammatory activity (5), interfered with the cholinergic contraction of rat ileum (6), induced relaxation in rat uterus (7), and antagonized α_1 -adrenoceptors and voltage-operated Ca^{2+} channels in rat aorta (8). Recently, Chuliá and co-workers (9) reported that *S*-(+)-boldine acts as an α_1 -adrenoceptor antagonist with little effect on KCl-induced contraction in rat aorta and does not modify the acetylcholine (ACh) response in guinea pig trachea. *N*-Methylboldine was shown to be a neuromuscular blocking agent (10) but its mechanisms of action have not been examined.

In this study, we used the phrenic nerve-diaphragm to study the effect of boldine on neuromuscular transmission. We found that boldine blocked the neuromuscular action reversibly by direct interaction with the postsynaptic acetylcholine receptor.

MATERIALS AND METHODS

Materials

All chemicals, unless specified, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Boldine, isolated from *Peumus boldus* Mol., was purchased from Sigma and dissolved in DMSO for all experiments. The final DMSO concentration was kept at 0.1% and was used in each respective control experiment.

Mouse phrenic nerve-diaphragm preparation

Mice (ICR strain) of either sex, weighing 15–20 g, were used. The phrenic nerve-diaphragm preparation was isolated according to the method of Bülbring (11). The diaphragm preparation was placed in Krebs solution of the following composition: 118.5 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 , 25 mM $NaHCO_3$, 11.1 mM glucose and 2.5 mM $CaCl_2$, pH 7.4 and constantly gassed with 95% O_2 + 5% CO_2 at $37.0 \pm 0.5^\circ C$. Twitches of the diaphragm were elicited by either stimulation of the phrenic nerve (indirect stimulation) with a supramaximal rectangular pulse of 0.05-msec duration at 0.2 Hz or direct stimulation of the muscle with a pulse of 0.5 msec

in the absence or in the presence of 1×10^{-5} g/ml *d*-tubocurarine (d-TC) or $5 \mu\text{M}$ neostigmine.

For chronic denervation, a section of about 1 cm of the left phrenic nerve was removed under pentobarbital anesthesia. The denervated diaphragm and the contralateral innervated one were isolated 10 days later. The muscle was loaded with a resting tension of 0.5 g, and the changes of tension were recorded via an isometric transducer (Model FT.03; Grass Instrument Co., Quincy, MA, USA) on a polygraph (Model RS3200; Gould Instrument Co., Cleveland, OH, USA).

Intracellular recording of end plate potential

Intracellular recording of the diaphragm was performed according to the microelectrode techniques described by Fatt and Katz (12). The glass microelectrodes were filled with 3 M KCl and had a resistance in the range of 3–10 M Ω . The mouse diaphragm was placed in Krebs solution at $37.0 \pm 0.5^\circ\text{C}$ and constantly oxygenated

with 95% O₂ + 5% CO₂. The amplitude and frequency of miniature endplate potentials (m.e.p.p.) were recorded with a high impedance amplifier (Model FD223; WPI, Sarasota, FL, USA) and digitized by a digitizing unit (Neuro-Corder DR384; Cygnus Technology Inc., Delaware Water Gap, PA, USA) and stored on a video tape recorder for later playback on a Tektronix oscilloscope (Model 2221A; Tektronix, Wilsonville, OR, USA).

Acetylcholinesterase activity measurement

The enzymatic activity of acetylcholinesterase (AChE; EC 3.1.1.7) purified from electric eel (type V-S) was determined by the method of Ellman et al. (13) using acetylthiocholine iodide as substrate and 5,5'-dithio-(bis-2-nitrobenzoic acid) (DTNB) as a coupler. Briefly, 0.125 U AChE was incubated in 1 ml of 0.1 M phosphate buffer (pH 8.0) containing 0.3 mM DTNB and test compounds for 2 min at 37°C. The reaction was started by addition of 0.5 mM acetylthiocholine and activity measured spectro-

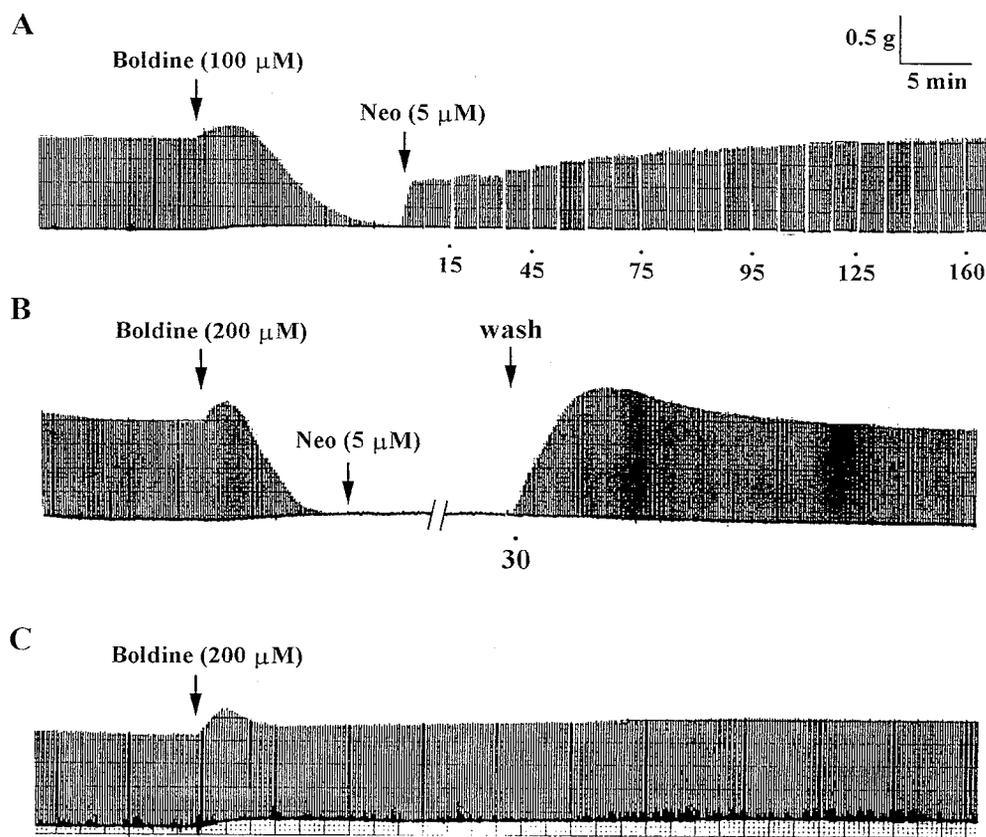


Fig. 1. Effect of boldine on the twitches evoked by either nerve stimulation or direct muscle stimulation. The isolated mouse phrenic nerve-diaphragm preparation was stimulated either indirectly on the nerve (A, B) or directly on the muscle in the presence of 1×10^{-5} g/ml *d*-tubocurarine (d-TC) (C) in normal Krebs according to the procedures described in the Materials and Methods. Note that boldine (B) at $100 \mu\text{M}$ produced, after an initial period of twitch augmentation, depression of the twitch contraction, which was reversed by $5 \mu\text{M}$ neostigmine (Neo) (A). Boldine caused reversible neuromuscular blockade at $200 \mu\text{M}$ (B); however, it did not block the twitches evoked by direct stimulation (C). Boldine at $200 \mu\text{M}$ caused a slight increase of resting tension either in the absence (B) or in the presence of d-TC (C).

photometrically at 412 nm as a function of time with a spectrophotometer (Model DU-650; Beckman, Fullerton, CA, USA). All operations were performed in the dark due to the photosensitivity of the AChE. The K_m value for AChE under the assay condition used was found to be 0.3 ± 0.02 mM.

Statistical analyses

The statistical significance of difference between the control and drug-treated preparations was evaluated by Student's *t*-test. A *P* value of 0.05 or less was considered statistically significant.

RESULTS

Inhibition of nerve-evoked twitches in the diaphragm by boldine

The effects of boldine on the neuromuscular junction and skeletal muscle were studied with phrenic nerve-diaphragm stimulated either indirectly at the phrenic-nerve (nerve-evoked) or directly to the skeletal muscle (muscle-evoked). Boldine produced, after an initial period of twitch augmentation, paralysis of the indirectly stimulated skeletal muscle (Fig. 1: A and B). The neuromuscular blockade by $100 \mu\text{M}$ (Fig. 1A), but not $200 \mu\text{M}$ (Fig. 1B), boldine was restored by $5 \mu\text{M}$ neostigmine, an anticholinesterase. A similar effect was seen with physostigmine (data not shown). The effect of boldine could be reversed by washout with Krebs solution (Fig. 1B). In contrast to the inhibitory effect on indirect stimulation, the twitches evoked by direct stimulation of muscle were not inhibited by boldine at concentrations up to $200 \mu\text{M}$ (Fig. 1C). The twitches evoked directly were potentiated transiently by boldine (Fig. 1C) as in the case of the indirectly-evoked muscle, suggesting that this twitch potentiation by boldine might result from direct action on muscle cells. We have also found that boldine, at $200 \mu\text{M}$, caused a slight increase of resting tension (Fig. 1: B and C). Neither the twitch augmentation nor the increase of resting tension induced by boldine were inhibited by prior addition of d-TC and neostigmine (data not shown).

Boldine inhibited the ACh-induced contraction in denervated diaphragm

The effect of boldine was further investigated with the denervated mouse diaphragm, and typical traces are shown in Fig. 2A. Addition of $30 \mu\text{M}$ ACh evoked a contraction of the denervated diaphragm, which was inhibited by prior addition of boldine (Fig. 2A). The ACh-induced contraction in the denervated diaphragm was inhibited by boldine in a dose-dependent manner (Fig. 2B) with an IC_{50} value of $13.5 \mu\text{M}$. These data suggested that boldine might act directly on the ACh receptor.

Previously, Erhardt and Soine (10) showed that *N*-methylboldine could inhibit AChE activity. By directly measuring the activity of AChE purified from electric eel, we found that boldine dose-dependently inhibited the AChE activity with an IC_{50} value of $200 \pm 25 \mu\text{M}$.

Antagonist action of boldine on ACh receptor

As shown in Fig. 3, 1×10^{-6} g/ml α -bungarotoxin (α -BuTx) inhibited the nerve-evoked twitches of diaphragm in an irreversible manner (Fig. 3A). Pretreatment with $5 \mu\text{M}$ d-TC protected the mouse diaphragm from the irreversible neuromuscular blocking action of α -BuTx (Fig. 3B). Similar to d-TC, boldine protected the mouse

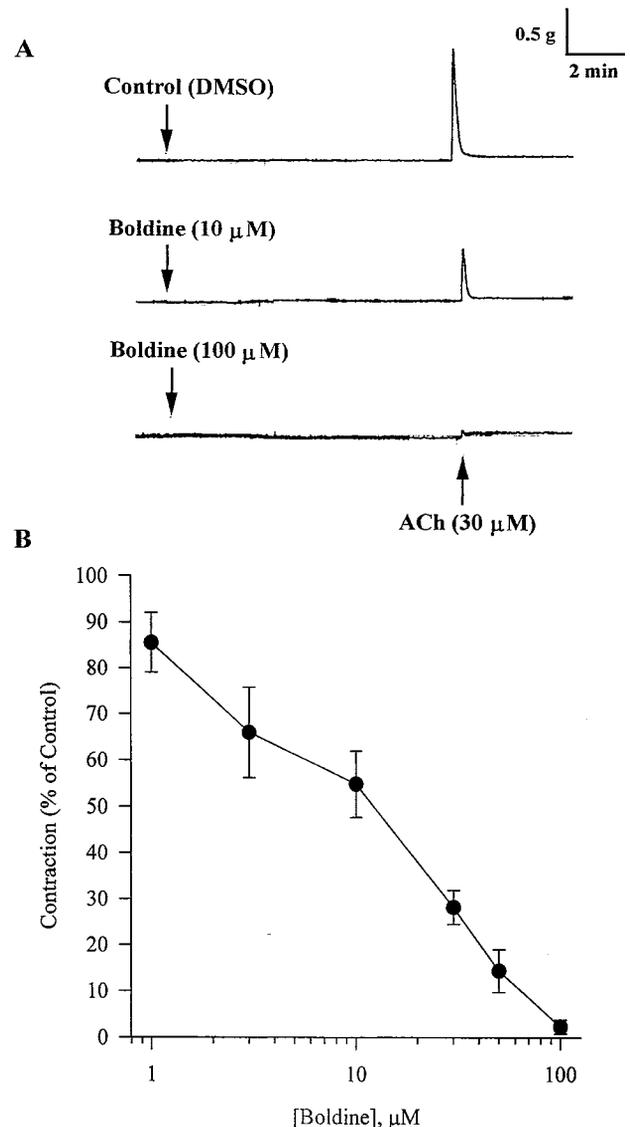


Fig. 2. Inhibition of acetylcholine (ACh)-induced contraction in denervated-diaphragm. Boldine inhibited the contraction evoked by $30 \mu\text{M}$ ACh (A) in a dose-dependent manner (B). The data are expressed as the mean \pm S.E.M. ($n=6$).

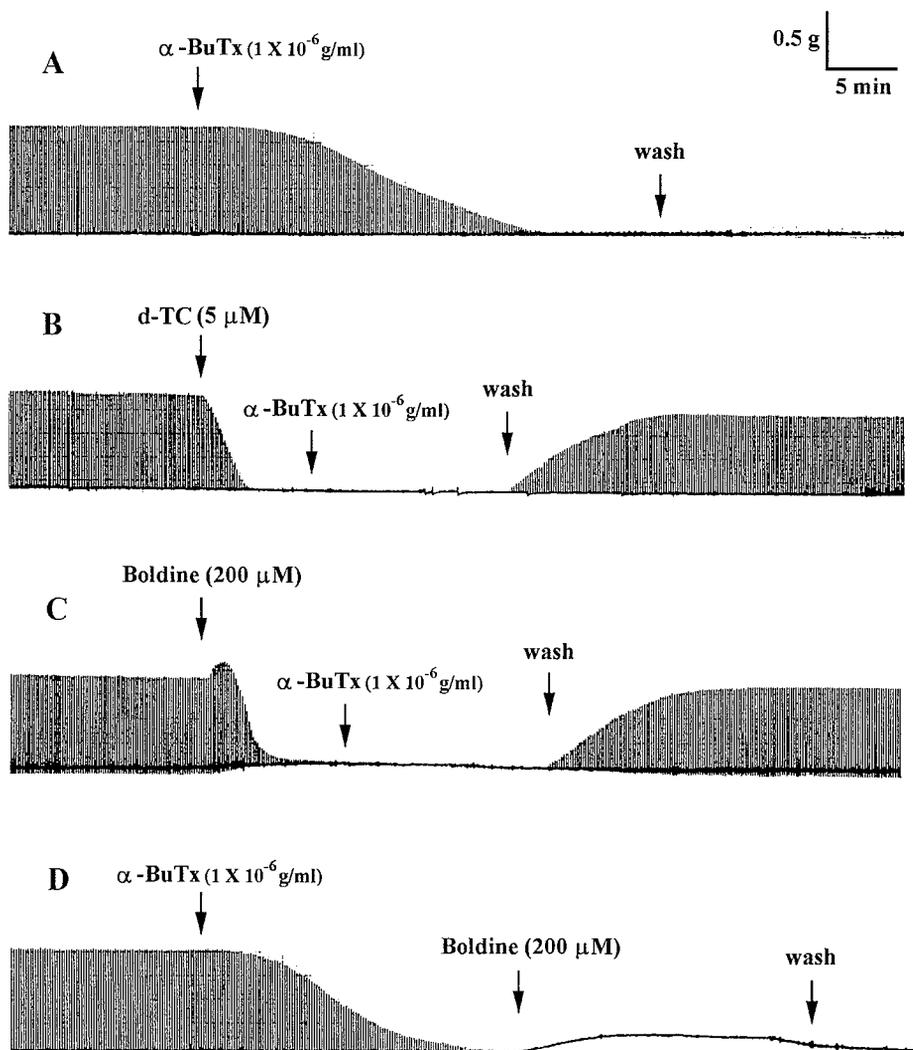


Fig. 3. Reversal of the neuromuscular blocking action of α -bungarotoxin (α -BuTx) by *d*-tubocurarine (*d*-TC) and boldine. The phrenic nerve of the mouse was stimulated, and the twitches were recorded isometrically as described in the Materials and Methods. The twitches were blocked irreversibly by α -BuTx (1×10^{-6} g/ml) alone (A), while pretreatment with either *d*-TC (5 μ M) (B) or boldine (200 μ M) (C) restored the twitches after washout with Krebs solution. The action of α -BuTx was not reversed by boldine when added after the neuromuscular blockade caused by α -BuTx (D). Note that boldine, as described previously, caused an increase of resting tension.

diaphragm from the neuromuscular blocking action of α -BuTx when added prior to (Fig. 3C), but not after (Fig. 3D), the addition of α -BuTx.

Effect of boldine on m.e.p.p.

Effect of boldine on m.e.p.p. was investigated, and the results were shown in Fig. 4. The m.e.p.p. of the control diaphragm (Fig. 4A) was inhibited by 50 μ M boldine (Fig. 4B). The amplitude, but not the frequency, of m.e.p.p. was inhibited significantly by boldine (B in Fig. 4C), which was reversed by washout with Krebs solution (W in Fig. 4C). The m.e.p.p. was completely inhibited by boldine at concentrations greater than 100 μ M.

DISCUSSION

We have shown that boldine, the major alkaloid in the leaves and bark of Boldo (*Peumus boldus* Mol.), exerted several distinct actions on skeletal muscle. In addition to the initial augmentation of either nerve- or muscle-evoked twitches, boldine preferentially inhibited the nerve-evoked twitches of the mouse diaphragm and left the muscle-evoked twitches unaffected, suggesting that boldine might either inhibit ACh release from motor nerve terminals or antagonize the postsynaptic ACh receptors. The twitch inhibition could be restored by washout with Krebs solution or by neostigmine, an anticholinesterase

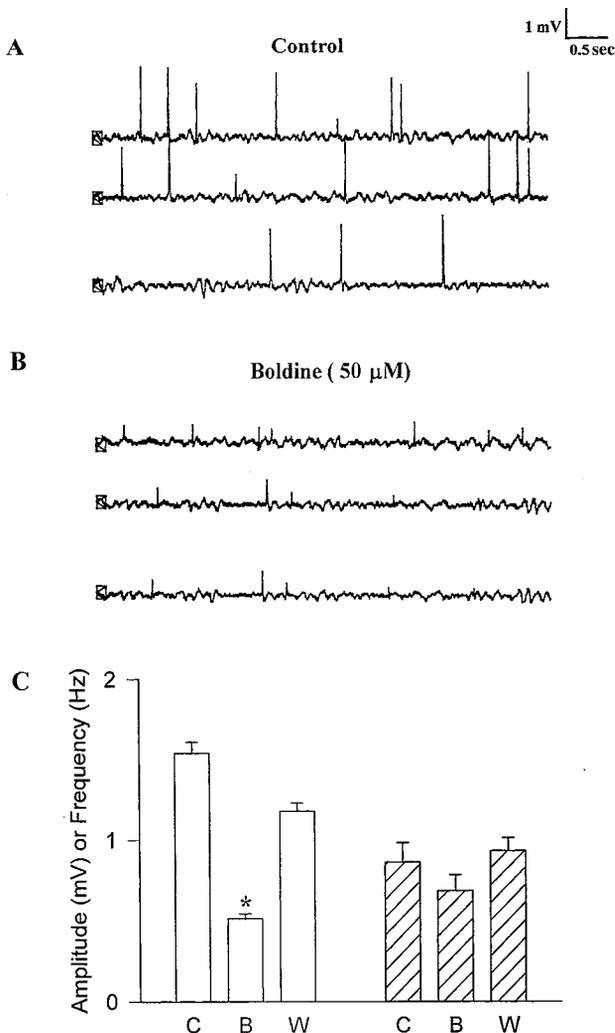


Fig. 4. Reduction of m.e.p.p amplitude by boldine. A: the control m.e.p.p. B: the m.e.p.p of isolated diaphragm in the presence of 50 μ M boldine. C: the amplitude (\square) but not the frequency (hatched) of control (C) m.e.p.p. was inhibited significantly by 50 μ M boldine (B), which could be reversed by washout (W). The data are expressed as the mean \pm S.E.M. ($n=3$). * $P < 0.01$, compared with the control.

agent. In addition to its ability to produce a neuromuscular blockade, we have found that boldine can induce twitch augmentation of nerve-evoked twitches, which might partly be due to the inhibition of AChE by boldine. However, we have found that indirectly and directly evoked twitches were both augmented initially and neither neostigmine nor d-TC affected the boldine-induced augmentation of twitch contraction. These results indicated that twitch potentiation of boldine might result from a direct action on muscle cells. The direct effect of boldine on muscle cells was further evidenced by the fact that boldine, at 200 μ M, caused a slight but consistent increase of resting tension. The underlying mechanisms through which boldine induced twitch augmentation and

muscle contracture are currently under investigation.

The inhibition of postsynaptic ACh receptors by boldine was further evidenced by the fact that boldine dose-dependently inhibited the ACh-induced contraction in the denervated diaphragm and the amplitude of m.e.p.p. We also showed that boldine, like d-TC, could reverse the neuromuscular blocking action of α -BuTx, suggesting that they might bind competitively to the same site on the ACh receptors of skeletal muscle. Recently, Chuliá et al. (9) showed that boldine antagonized the α_1 -adrenoceptor of guinea pig aorta, but had no effect on ACh-induced contraction of the trachea. These data suggested that boldine might have higher specificity towards the nicotinic ACh receptor than the muscarinic ACh receptor.

Ivorra and co-workers (7, 8) have shown that boldine blocked the voltage-dependent Ca^{2+} channel of the uterus and aorta. We have found that pretreatment with boldine inhibited the K^+ -induced contracture dose-dependently with an IC_{50} value of 7 μ M, indicating that boldine at lower concentrations is also an effective Ca^{2+} channel blocker of skeletal muscle (unpublished results). It was shown that high K^+ -induced contractures could be eliminated by removing Ca^{2+} from the extracellular spaces (14) or blocked by a dihydropyridine Ca^{2+} channel antagonist like nitrendipine (15). Such results suggest that the entrance of external Ca^{2+} via the L-type Ca^{2+} channels is required for excitation-contraction coupling in skeletal muscle during high K^+ -induced contractures but not for twitches (15). This could explain why we did not observe the inhibition of muscle-evoked twitches by boldine.

Although we have presented evidence showing that the neuromuscular blocking action exerted by boldine might be due to the postsynaptic inhibition of ACh channel, the possible inhibitory effect of boldine on presynaptic ACh release can not be excluded. The entry of Ca^{2+} into the presynaptic nerve terminals through voltage-gated Ca^{2+} channels was recognized to play an essential role in evoked transmitter release at the neuromuscular junction. There are at least four types of voltage-dependent Ca^{2+} channels that have been demonstrated in neurons, L-, N-, T- and P-channels (16, 17), and much work has been done to assign distinct functions to specific channel types. The N-type channel plays a role of neurotransmitter release in a number of preparations, including central, autonomic and peripheral nervous systems (18–22). However, the P-type Ca^{2+} channel mediates transmitter release in cerebellar Purkinje cells (23) and mammalian neuromuscular junction (24), including mouse phrenic nerve-diaphragm. Although we have shown here that boldine can block the L-type Ca^{2+} channel of skeletal muscle, whether boldine can also affect the P-type Ca^{2+} channel at nerve terminals and result in the inhibition of presynaptic ACh release needs further investigation.

Acknowledgment

This work was supported by a research grant from the National Science Council, Taiwan (NSC85-2331-B-002-288).

REFERENCES

- 1 Van Hulle C, Braeckman P and Van Severen R: Influence of the preparation technique on the boldine content of boldo dry extract. *J Pharm Belg* **38**, 97–100 (1983) (in French)
- 2 Lanhers MC, Joyeux M, Soulimani R, Fleurentin J, Sayag M, Mortier F, Younos C and Pelt J-M: Hepatoprotective and anti-inflammatory effects of a traditional medicinal plant of Chile, *Peumus boldus*. *Planta Med* **57**, 110–115 (1991)
- 3 Speisky H and Cassels BK: Boldo and boldine: an emerging case of natural drug development. *Pharmacol Res* **29**, 1–12 (1994)
- 4 Speisky H, Cassels B, Lissi EA and Videla LA: Antioxidant properties of the alkaloid boldine in systems undergoing lipid peroxidation and enzyme inactivation. *Biochem Pharmacol* **41**, 1575–1581 (1991)
- 5 Backhouse N, Delporte C, Givernau M, Cassels BK, Valenzuela A and Speisky H: Anti-inflammatory and antipyretic effects of boldine. *Agents Actions* **42**, 114–117 (1994)
- 6 Speisky H, Squella JA and Núñez-Vergara LJ: Activity of boldine on rat ileum. *Planta Med* **57**, 519–522 (1991)
- 7 Ivorra MD, Martínez F, Serrano A and D'Ocón MP: Different mechanism of relaxation induced by aporphine alkaloids in rat uterus. *J Pharm Pharmacol* **45**, 439–443 (1993)
- 8 Ivorra MD, Chuliá S, Lugnier C and D'Ocón MP: Selective action of two aporphines at α_1 -adrenoceptors and potential-operated Ca^{2+} channels. *Eur J Pharmacol* **231**, 165–174 (1993)
- 9 Chuliá S, Moreau J, Naline E, Noguera MA, Ivorra MD, D'Ocón MP and Advenier C: The effect of *S*-(+)-boldine on the α_1 -adrenoceptor of the guinea-pig aorta. *Br J Pharmacol* **119**, 1305–1312 (1996)
- 10 Erhardt PW and Soine TO: Stereochemical preferences for curarimimetic neuromuscular junction blockade I: enantiomeric monoquatary amines as probes. *J Pharm Sci* **64**, 53–70 (1975)
- 11 Bülbring E: Observations on the isolated phrenic nerve diaphragm preparation of the rat. *Br J Pharmacol* **1**, 38–61 (1946)
- 12 Fatt P and Katz B: An analysis of the endplate potential recorded with an intracellular electrode. *J Physiol (Lond)* **115**, 320–370 (1951)
- 13 Ellman GL, Kourtney KD, Andres V Jr and Featherstone RM: A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* **7**, 88–96 (1961)
- 14 Frank GB: Effects of changes in extracellular calcium concentration on the potassium-induced contracture of frog's skeletal muscle. *J Physiol (Lond)* **151**, 518–538 (1960)
- 15 Frank GB: Dihydropyridine calcium channel antagonists block and agonists potentiate high potassium contractures but not twitches in frog skeletal muscle. *Jpn J Physiol* **40**, 205–224 (1990)
- 16 Bertolin M and Llinas RF: The central role of voltage-activated and receptor-operated calcium channels in neuronal cells. *Annu Rev Pharmacol* **32**, 399–421 (1992)
- 17 Tsien RW, Lipscombe D, Madison DW, Bley KR and Fox AP: Multiple types of neuronal calcium channels and their selective modulation. *Trends Neurosci* **11**, 431–438 (1988)
- 18 Dooley DJ, Lupp A, Hertting G and Osswald H: ω -Conotoxin GVIV and pharmacological modulation of hippocampal noradrenaline release. *Eur J Pharmacol* **148**, 261–267 (1988)
- 19 Hirning LD, Fox AF, McCleskey EW, Olivera BM, Thayer SA, Miller RJ and Tsien RW: Dominant role of N-type Ca channels in evoked release of norepinephrine from sympathetic neurons. *Science* **239**, 57–60 (1988)
- 20 Horne AL and Kemp JA: Effect of ω -conotoxin GVIV on synaptic transmission in the nucleus accumbens and hippocampus of the rat in vitro. *Br J Pharmacol* **103**, 1733–1739 (1991)
- 21 Mohy El-Din MM and Malik KU: Differential effect of ω -conotoxin on release of the adrenergic transmitter and the vasoconstrictor response to noradrenaline in the rat isolated kidney. *Br J Pharmacol* **94**, 355–362 (1988)
- 22 Yoshikami D, Bagabaldo Z and Olivera BM: The inhibitory effects of omega-conotoxins on Ca channels and synapses. *Ann NY Acad Sci* **560**, 230–248 (1989)
- 23 Llinas R, Sugimori M, Lin J-W and Cherksey B: Blocking and isolation of a calcium channel from neurons in mammals and cephalopods utilizing a toxin fraction (FTX) from funnel-web spider poison. *Proc Natl Acad Sci USA* **84**, 4327–4331 (1989)
- 24 Uchitel OD, Protti DA, Sanchez V, Cherksey BD, Sugimori M and Llinas R: P-type voltage-dependent calcium channel mediates presynaptic calcium influx and transmitter release in mammalian synapses. *Proc Natl Acad Sci USA* **89**, 3330–3333 (1992)