

Ephedrine Blocks Rat Sciatic Nerve In Vivo and Sodium Channels In Vitro

Yu-Chun Hung, M.D.,* Yi-Chuan Kau, M.D.,† Anthony M. Zizza, B.A.,‡ Thomas Edrich, M.D., Ph.D.,§ David Zurakowski, Ph.D.,|| Robert R. Myers, Ph.D.,# Ging Kuo Wang, Ph.D.,** Peter Gerner, M.D.††

Background: The sympathomimetic drug ephedrine has been used intrathecally as the sole local anesthetic for labor and delivery. Because ephedrine may be a useful adjuvant to local anesthetics, the authors investigated the local anesthetic properties of ephedrine in a rat sciatic nerve block model and the underlying mechanism in cultured cells stably expressing Na⁺ channels.

Methods: After approval of the animal protocol, the sciatic nerves of anesthetized rats were exposed by lateral incision of the thighs. 0.2 ml ephedrine at 0.25, 1, 2.5, or 5% and/or bupivacaine at 0.125% was injected, and the wound was closed. Motor and sensory/nociceptive functions were evaluated by the force achieved by pushing against a balance and the reaction to pinch, respectively. The whole cell configuration of the patch clamp technique was used to record Na⁺ currents from human embryonal kidney cells stably transfected with Na_v1.4 channels.

Results: The nociception blockade was significantly longer than the motor blockade at test doses of 2.5 and 5% of ephedrine, or when 1% ephedrine was combined with 0.125% bupivacaine (analysis of variance with repeated measures, $P < 0.001$, $n = 8/\text{group}$). *In vitro*, the 50% inhibitory concentrations of ephedrine at -150 and -60 mV were $1,043 \pm 70$ and 473 ± 13 μM , respectively. High-frequency stimulation revealed a use-dependent block of 18%, similar to most local anesthetics.

Conclusions: Because ephedrine's properties are at least partly due to Na⁺ channel blockade, detailed histopathologic investigations are justified to determine the potential of ephedrine as an adjuvant to clinically used local anesthetics.

EPHEDRINE is an adrenergic agonist used by anesthesiologists on a daily basis to increase blood pressure and heart rate, particularly for maternal hypotension associated with spinal anesthesia.^{1–3} It is currently approved by the U.S. Food and Drug Administration for the treatment of nasal congestion, asthma, and hypotension arising from spinal blockade. Ephedrine's cardiovascular stimulatory and bronchodilatory effects are thought to be mediated indirectly by releasing norepinephrine from

sympathetic neurons, but ephedrine has also been shown to act as a direct sympathomimetic drug.^{4,5}

These adrenergic properties prompted us to reexamine ephedrine as a potential adjuvant for clinical and investigational local anesthetics (LAs). As expected, a review of the literature revealed that ephedrine, because of its vasoconstrictive properties, was coinjected with LAs (*i.e.*, pontocaine) to decrease the resorption of LAs and thereby prolong their duration.^{6,7} However, and to our surprise, we found that ephedrine was used as a sole LA agent more than half a century ago. In fact, the first volume of this journal, published in July 1940, contained an article entitled "The Local Anesthetic Properties of Ephedrine Hydrochloride." This article concluded that "Ephedrine HCl, in concentration of five percent, is capable of blocking the sciatic nerve of the frog" (an excised sciatic nerve and gastrocnemius muscle model was used).⁸ Intrathecal ephedrine (50 mg) had also been used as the sole agent even for obstetric delivery in the 1940s.⁹

To evaluate ephedrine as a potential adjuvant for clinical and investigational LAs, we (1) assessed whether ephedrine provides dose-dependent reversible block of motor and sensory/nociceptive functions in a rat sciatic nerve block model without degenerative histologic changes and (2) investigated ephedrine's mechanism of nerve blockade by determining its Na⁺ channel-blocking capabilities in cultured cells stably expressing Na⁺ channels.

Materials and Methods

Drugs

Ephedrine sulfate was purchased from Abbott Laboratories, Inc. (North Chicago, IL), and bupivacaine hydrochloride was a gift from AstraZeneca USA, Inc. (Westborough, MA). For the sciatic nerve blockade, ephedrine at 0.25% (5.8 mM), 1% (23.3 mM), 2.5% (58.3 mM), and 5% (116.7 mM) and bupivacaine at 0.125% (3.8 mM) were dissolved in 0.9% sodium chloride. Upon local injection, the relatively low pH of these pure solutions (pH range, 4.9–6.5) is likely to be buffered quickly by the tissue fluid, which has a pH of 7.4. For the electrophysiologic experiments, ephedrine sulfate was dissolved in dimethyl sulfoxide at 100 mM and was diluted shortly before the experiments.

Subfascial Sciatic Nerve Injections

The protocol for animal experimentation was reviewed and approved by the Harvard Medical Area

* Attending Anesthesiologist, Department of Anesthesiology, Mackay Memorial Hospital, Taipei, Taiwan. † Department of Anesthesiology, Chang Gung Memorial Hospital, Taoyuan, Taiwan. ‡ Research Assistant, § Research Fellow, ** Associate Professor, †† Assistant Professor, Pain Research Center, Department of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts. || Director of Biostatistics, Children's Hospital and Harvard Medical School, Boston, Massachusetts. # Professor of Anesthesiology and Pathology, University of California, San Diego, California.

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Address reprint requests to Dr. Gerner: Department of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women's Hospital, 75 Francis Street, Boston, Massachusetts 02115. Address electronic mail to: pgerner@partners.org. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

Standing Committee on Animals (Boston, Massachusetts). Female Sprague-Dawley rats were purchased from Charles River Laboratory (Wilmington, MA) and kept in the animal housing facilities at Brigham and Women's Hospital, with controlled relative humidity (20–30%), at room temperature (24°C), and in a 12-h (6:00 AM to 6:00 PM) light–dark cycle. Rats were handled before the procedure to familiarize them with the experimental environment and to minimize stress-induced analgesia. At the time of injections, animals weighed 200–250 g and showed no signs of neurobehavioral impairment. The experimenter was blinded to the drug and concentration used. For subfascial sciatic nerve blockade, rats were anesthetized by inhalation of 1–2% sevoflurane, and 2.5 mg ketamine and 1 mg xylazine were injected subcutaneously. After rats were anesthetized, the sciatic nerves were exposed by lateral incision of the thighs and division of the superficial fascia and muscle. With a 30-gauge needle attached to a tuberculin syringe, 0.2 ml of the test dose was injected directly beneath the clear fascia surrounding the nerve but outside the perineurium, proximal to the sciatic bifurcation. The test doses comprised 0.25, 1, 2.5, or 5% ephedrine and 0.125% bupivacaine, with an additional group comprising the combination of 1% ephedrine and 0.125% bupivacaine ($n = 8/\text{group}$). The superficial muscle layer was sutured with 4-0 silk, and the wound was closed as described.¹⁰

Neurobehavioral Examination

The neurobehavioral examination, modified from previous reports,¹¹ focused on motor function and nociception. Initially, rats were examined 30 min after drug administration, then at 15-min intervals until 120 min, and at 30-min intervals until complete recovery.

Briefly, we evaluated motor function by measuring the extensor postural thrust of the hind limbs by holding the rat upright with the hind limb extended so that the distal metatarsus and toes supported the animal's weight, thereby measuring the extensor thrust as the gram force applied to a digital platform balance (Ohaus Lopro; Fisher Scientific, Florham Park, NJ). The reduction in this force, representing reduced extensor muscle contraction due to motor blockade, was calculated as a percentage of the control force (preinjection control value range was 130–165 g). The percentage value was assigned a score: 0 = no block or baseline; 1 = minimal block, force between 100% and 50% of preinjection control value; 2 = moderate block; force between 50% of the preinjection control value and 20 g (approximately 20 g is the weight of the flaccid limb); 3 = complete block, force of 20 g or less.

We evaluated nociception by the withdrawal reflex or vocalization to pinch of a skin fold over the lateral metatarsus (cutaneous pain) and of the distal phalanx of the fifth toe (deep pain). We graded the combination of

withdrawal reflex and vocalization on a scale of 0–3, as above, and repeated the examination three times; the average was used.

Cell Culture

Cultures of rat clonal pituitary GH₃ cells as well as human embryonal kidney cells stably transfected with rat Na_v1.4 Na⁺ channels were split once a week and maintained in Dulbecco modified Eagle medium (Hyclon Labs, Logan, UT) supplemented with taurine (1%), penicillin–streptomycin (1%), hydroxyethyl-piperazineethane sulfonic acid, HEPES (20 mM), and heat-inactivated fetal bovine serum (10%), as described.¹² The 35-mm culture dishes in which the cells were grown also were used as recording chambers. Because ephedrine dose–response studies with rat GH₃ cells expressing endogenous neuronal Na⁺ channel isoforms (Na_v 1.1, 1.2 and 1.3) indicated that the potency was similar to that of the skeletal muscle isoform (Na_v1.4), we chose human embryonal kidney cells stably expressing Na_v1.4 for all patch clamp studies for reasons of low maintenance and most consistent expression of Na⁺ channels.

Whole Cell Voltage Clamp Experiments

The whole cell configuration of the patch clamp technique¹³ was used to record macroscopic Na⁺ currents at room temperature (21°–23°C). The pipette electrodes had a resistance ranging from 0.8 to 1.2 MΩ. Command voltages were controlled by pCLAMP software (Axons Instruments, Inc., Foster City, CA) and delivered by a List-EPC7 patch clamp amplifier (List-Electronic, Darmstadt-Eberstadt, Germany). After the whole cell configuration was established, cells were dialyzed for 30 min before data were acquired. Data were filtered at 3 kHz, sampled at 50 kHz, collected, and stored with pCLAMP software. Leak and capacitance currents were subtracted by the P/4 protocol. Whole cell recordings were maintained for more than 1 h in this preparation with little or no rundown of the Na⁺ current. Pipette electrodes were filled with an internal solution containing 100 mM NaF, 30 mM NaCl, 10 mM EGTA, and 10 mM HEPES titrated with CsOH to a pH of 7.2. The external solution consisted of 85 mM choline Cl, 2 mM CaCl₂, 65 mM NaCl, and 10 mM HEPES titrated with TMA-OH to a pH of 7.4. These solutions create an outward Na⁺ current at +30 mV,¹² further reducing potential problems associated with series resistance errors. Whole cell recordings can be maintained for more than 1 h in this preparation with little or no rundown of the Na⁺ current.

Voltage-dependent blockade by ephedrine was determined by the application of a prepulse or conditioning pulse that is long enough to permit the drug–channel binding interaction to reach its steady state level. The potencies for the resting and inactivated states were determined by constructing dose–response curves at conditioning potentials of –150 and –60 mV, respec-

tively. Finally, we investigated a potential additional block (use-dependent block) by utilizing a high-frequency stimulation protocol.

Pathologic Evaluation

Pathologic evaluation was used to ascertain the absence of neurotoxicity, because this would prove extremely helpful in future larger scale studies for choosing the most appropriate concentrations to minimize neurotoxicity of drug combinations.

In a pilot study, the six rats from the 1% ephedrine group that had complete motor block, and therefore were most likely to show toxicity, were killed 7 days after receiving the test dose. The 1% ephedrine group was selected for two reasons: First, because the intention was to evaluate ephedrine as an adjuvant, lower drug concentrations were more clinically useful; and second, because of the well-known vasoconstrictive properties of ephedrine, we expected neurodegenerative changes of ischemic origin even at this relatively low concentration. Four rats from the 0.125% bupivacaine group were chosen as active controls because they displayed similar block properties.

The 10 sciatic nerves were excised under the anesthesia protocol used for surgery. We then killed the rats by giving an overdose of pentobarbital (70 mg/kg). For fixation, we placed the nerves, measuring approximately 2 cm long with the injection site in the middle, on a wooden stick and immersed them in 2.5% phosphate-buffered glutaraldehyde for 24 h. We then rinsed the nerves three times with phosphate buffer, postfixed them in 1% osmium tetroxide, dehydrated them in serial concentrations of alcohol, and embedded them in araldite according to the recommended procedure for neurotoxicologic tissue evaluation.¹⁴ We cut twenty 1- μ m-thick semithin sections from the central 2-mm block of each 6-mm-long segment for light microscopy and stained them with methylene blue, azure II. An observer (R. R. M.) who was unaware of the experimental groupings evaluated the tissue sections.

Statistical Analysis

Two-way analysis of variance (ANOVA) was applied to test for differences in nociceptive and motor sciatic nerve block duration between different doses of ephedrine using the F test. A repeated-measures ANOVA model was used to assess differences in duration between nociceptive and motor sciatic nerve blockade because the same animals were evaluated with respect to both nociceptive and motor scores.¹⁵ A mixed-model regression analysis was also performed to evaluate differences in nociceptive and motor block durations for 1% ephedrine combined with 0.125% bupivacaine.¹⁶ Because multiple comparisons were planned, we set a two-tailed value of $P < 0.01$ as the level for statistical significance to protect against type I errors (false positives). A power analysis

indicated that the sample size of eight animals per group provided 80% statistical power ($\beta = 0.2$) to detect significant differences in block duration using ANOVA among the four doses of ephedrine and in the ephedrine with bupivacaine combination (version 5.0, nQuery Advisor; Statistical Solutions, Boston, MA). We presented the data in all figures in terms of the mean and SEM because we tested the data at each dose within the drug groups and the combination for normality using the Kolmogorov-Smirnov goodness-of-fit statistic and found no significant departures from a normal distribution ($P > 0.10$ in each case). Because this check on normality was verified, we then chose to report means and SEMs and analyze the data using parametric statistical methods (ANOVA).

Results

Rat Sciatic Nerve Blockade

Nociceptive and Motor Sciatic Nerve Blockade by Ephedrine. All groups ($n = 8/\text{group}$) developed a dose-dependent degree of sciatic nerve blockade after subfascial injections of ephedrine (figs. 1A and B). No sign of any other type of neurobehavioral abnormality was detected, and all rats recovered completely, with no clinically detectable neurologic deficits.

Two-way ANOVA indicated that the nociception blockade was significantly longer for 5% *versus* 1% ephedrine ($F = 23.69, P < 0.0001$), 5% *versus* 2.5% ephedrine ($F = 26.81, P < 0.0001$), and 2.5% *versus* 1% ephedrine ($F = 7.96, P < 0.001$). In addition, the motor blockade was significantly longer for 5% *versus* 1% ephedrine ($F = 7.82, P < 0.001$), 5% *versus* 2.5% ephedrine ($F = 8.07, P < 0.001$), and 2.5% *versus* 1% ephedrine ($F = 2.63, P = 0.008$). In short, a highly significant dose-response relation showed a less rapid return to baseline for the higher test doses in both nociception and motor blockades. In addition, ANOVA with repeated measures indicated that the nociception blockade was significantly longer than the motor blockade at test doses of 2.5% ($F = 4.86, P < 0.001$) and 5% ephedrine ($F = 5.15, P < 0.001$). No significant differences were found in the duration of nociception and motor blockades at 0.25% or 1% doses of ephedrine ($P = 0.99$ for both).

Nociceptive and Motor Sciatic Nerve Blockade by Bupivacaine. Bupivacaine, 0.125%, did not produce a complete block in all animals, which seems consistent with clinical experience (fig. 1C). Two-way ANOVA with repeated measures indicated no significant difference between the nociceptive and motor block durations ($F = 0.27, P = 0.98$).

Nociceptive and Motor Sciatic Nerve Blockade by 1% Ephedrine Combined with 0.125% Bupivacaine. This combination produced an intensified nerve blockade, with more nociceptive than motor block (fig. 1D). Two-way ANOVA with repeated measures con-

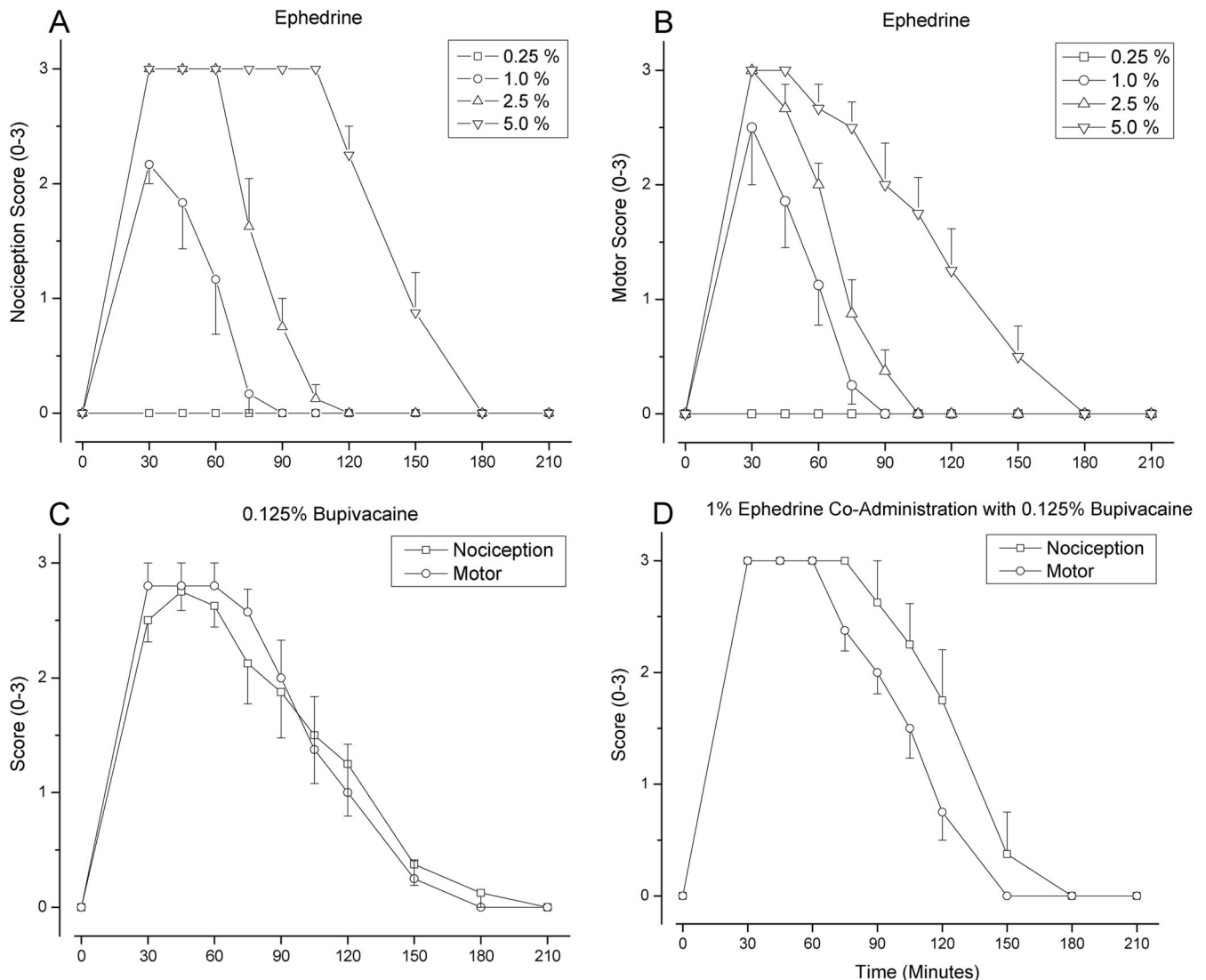


Fig. 1. Rat sciatic nerve blockade with ephedrine, bupivacaine, or a combination of both ($n = 8/\text{group}$; data are presented as mean \pm SEM). (A) Time course of nociceptive blockade by ephedrine administered to the sciatic nerve in rats at various concentrations. A score of 0 indicates no block or baseline, and a score of 3 indicates complete blockade of pain behavior by pinch (no withdrawal or vocalization). Rats in the 0.25% (5.8 mM) group did not have development of any block, and not all animals in the 1% (23.3 mM) group had development of complete block. (B) Time course of motor blockade by ephedrine. Similar to the grading of nociceptive blockade, a score of 0 indicates no block or baseline, and a score of 3 indicates complete paralysis (flaccid limb), *i.e.*, the rat is unable to push the tested hind limb against the balance. (C) Time course of nociceptive and motor blockade by bupivacaine to the sciatic nerve in rats at a concentration of 0.125% (3.8 mM). (D) Time course of nociceptive and motor blockade by 0.125% bupivacaine combined with 1% ephedrine. A statistically significantly prolonged sensory/nociceptive block over motor block is present during the recovery phase from complete blockade to baseline.

firmly a significantly longer nociceptive block than motor block ($F = 3.97$, $P < 0.001$).

Voltage Clamp Experiments

Voltage-dependent Inactivation of Na^+ Channels by Ephedrine. This experiment was performed to determine the steady state (h_{∞}) inactivation of Na^+ channels, indicating that a prepulse of -150 mV is appropriate to elicit dose-response curves for the resting state and -60 mV for the inactivated state (fig. 2A). The addition of ephedrine to the bath solution produces a 7.7-mV left shift of the inactivation curve. Such a left shift of the steady state inactivation is typical for LAs.

Potency of Ephedrine for the Resting and Inactivated States. Dose-response curves were constructed at conditioning potentials of -150 and -60 mV, respectively (fig. 2B). The 50% inhibitory concentrations (IC_{50} s) of ephedrine at -150 and -60 mV are estimated to be 1043 ± 70 and $473 \pm 13 \mu\text{M}$, respectively. Of note, when using GH_3 cells, we found a similar IC_{50} of $716 \pm 27 \mu\text{M}$ at -150 mV.

Use-dependent Blockade by Ephedrine. In addition to a tonic block exhibited when the cell is stimulated infrequently (0.03 Hz or once every 30 s as above for obtaining dose-response curves), the cell also exhibits a use-dependent block when it is stimulated fre-

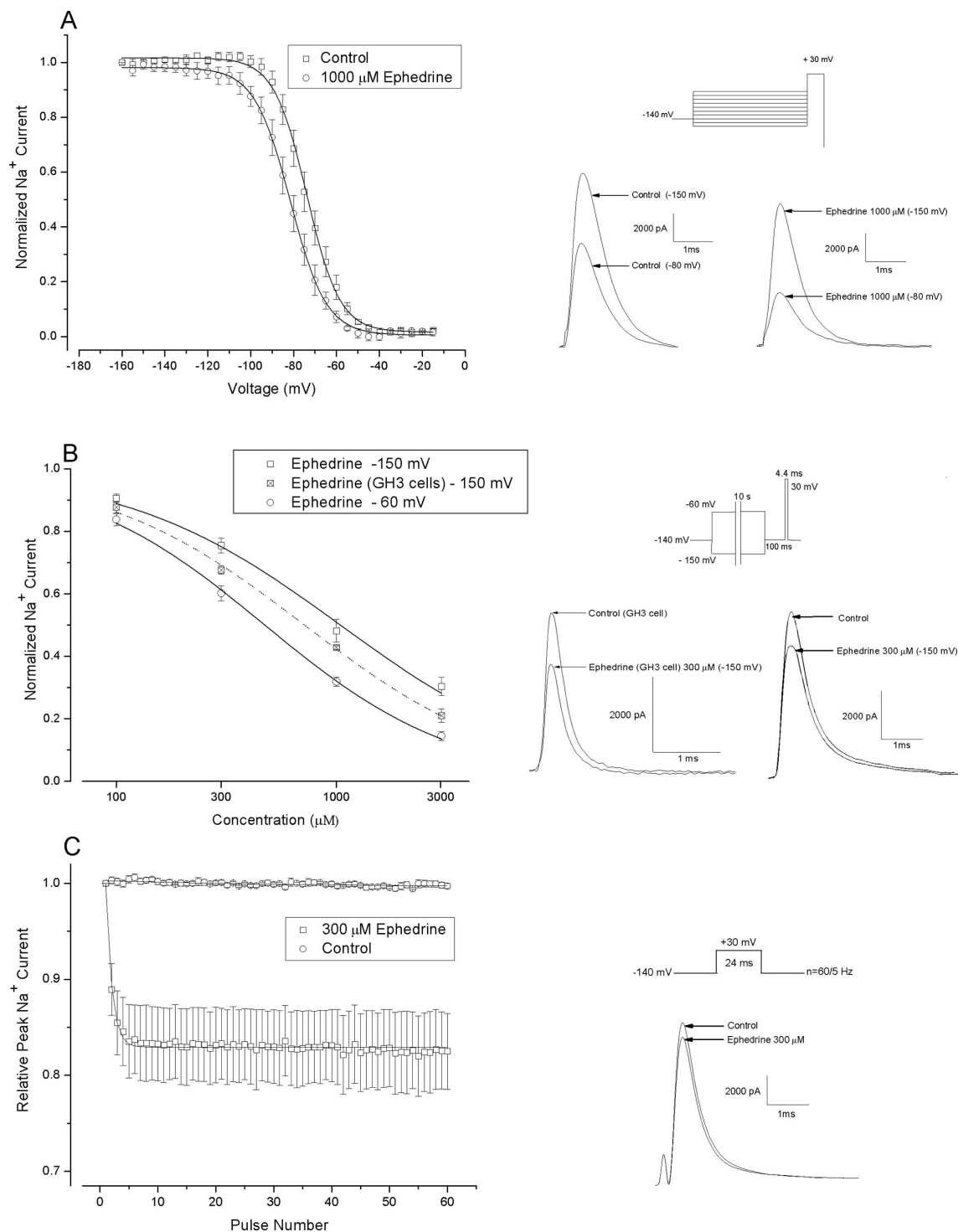


Fig. 2. Na⁺ current inhibition by ephedrine ($n = 5$ cells/group; data are presented as mean \pm SEM). The respective pulse protocol is inserted above the representative tracings. (A) Voltage-dependent block of Na⁺ channels by ephedrine. Conditioning prepulses ranging in amplitude from -160 to -15 mV were applied. Na⁺ currents were evoked by the delivery of the test pulse to $+30$ mV. Normalized Na⁺ current in the absence (control) or presence of $1,000 \mu\text{M}$ ephedrine was plotted against conditioning prepulse potential. Data were fitted well with a Boltzmann function. The average $V_{0.5}$ value (50% availabilities) and K_E (a slope factor) values for the fitted Boltzmann functions were -81.5 ± 0.2 and -73.8 ± 0.3 mV for control and ephedrine, respectively, and 8.3 ± 0.2 and 7.6 ± 0.2 mV, respectively. (B) Dose-response curves for hyperpolarized and depolarized sodium channels. The hyperpolarized state affinity for ephedrine on Na⁺ channels was measured with a prepulse of -150 mV for 10 s, and the depolarized state affinity was measured with prepulse of -60 mV for 10 s. Pulses were delivered at 30-s intervals. Lines connecting data points represent fits to the data with the Hill equation. The similarities of blocking properties between HEK cells expressing Na_v1.4 (skeletal muscle Na⁺ channel isoform) and GH₃ cells expressing Na_v1.1, 1.2, and 1.3 (neuronal Na⁺ channel isoforms) are illustrated by the dose-response curve of ephedrine at -150 mV (dashed line) as well as in the representative tracing. (C) Use-dependent block of Na⁺ channels by ephedrine. A test pulse of -30 mV was evoked for 24 ms at 5 Hz. Lines drawn through the data points are the best fit of a single exponential function.

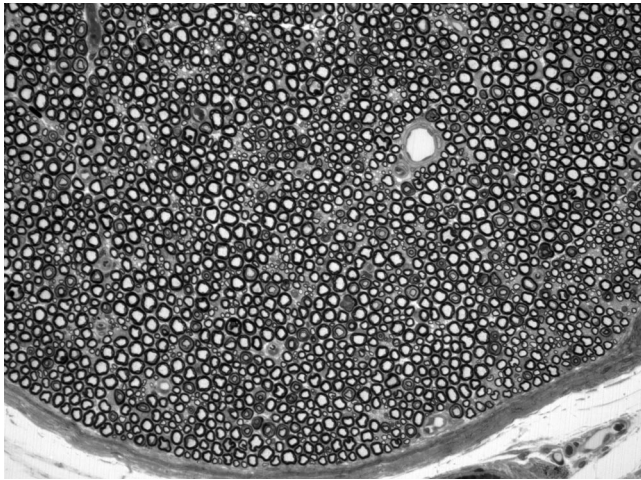


Fig. 3. Histopathology of rat sciatic nerve treated with 1% (23.3 mM) ephedrine (n = 6). The nerve is normal except for occasional activated Schwann cells in the absence of endoneurial edema. The endothelial cells are also normal, although the epineurium is slightly thickened. These nerve sections do not differ appreciably from controls (0.125% or 3.8 mM bupivacaine) at the light microscopic level.

quently (fig. 2C). Repetitive depolarization of cells at a frequency of 5 Hz produced a use-dependent block (approximately 18%) in the presence of ephedrine compared with no block in control cells. The time course of this use-dependent block was fitted by a single exponential function with a rate constant of 1.05 per pulse. Therefore, as shown previously for most LAs in various cell models, ephedrine also exhibited use-dependent block of $\text{Na}_v1.4 \text{ Na}^+$ channels.

Histopathology

Rat Sciatic Nerve Treated with 1% Ephedrine (n = 6). The nerve was normal except for occasional activated Schwann cells. There was no endoneurial edema, and the endothelial cells were normal. The epineurium was slightly thickened (fig. 3). Rats treated with 0.125% bupivacaine (control, n = 4) showed normal peripheral nerve histology.

Discussion

Ephedrine provides (1) dose-dependent reversible rat sciatic nerve blockade without major histopathology at 1% and (2) Na^+ channel blockade in cultured cells stably expressing Na^+ channels, which is at least part of its mechanism of action as an LA agent.

Although no formal dose-response curves were constructed, it can be easily estimated that the potency of ephedrine is much lower than that of bupivacaine (approximately 8 times less) in rat sciatic nerve blockade. This relatively high dosage of ephedrine necessary to block the nerve caused us to consider that the nerve blockade might be due, at least in part, to acute ischemia. However, the histologic findings demonstrated no

significant pathologic changes in the animals that had complete motor blockade from the 1% ephedrine group. The vasoconstrictive properties of ephedrine also seem rather mild, because studies performed approximately 50 yr ago showed controversial results of ephedrine in prolonging spinal anesthesia when added to LAs,^{6,7,17,18} such as pontocaine. For example, Potter and Whitacre⁶ reported more than 500 cases in which the addition of ephedrine sulfate increased the effectiveness of pontocaine for spinal anesthesia. Taylor¹⁷ suggested that ephedrine sulfate increases block duration of pontocaine and decreases the dosage of pontocaine needed. However, a study published in 1949 showed 50 mg ephedrine sulfate was able only to intensify the effects of pontocaine, not to prolong the sensory and motor blockade.⁷ Because newer LAs are known to decrease nerve blood flow by up to 70% without any signs of degeneration of ischemic origin,¹⁹⁻²² vasoconstriction induced by ephedrine might not be as detrimental as feared, probably because there is a significantly changed supply-demand ratio when the nerve is in a blocked state. Nevertheless, a limitation of our study is that a laser Doppler flowmeter was not used to evaluate nerve blood flow as described.²³ If such measurements showed that ephedrine does not significantly decrease nerve blood flow, that would increase the safety assessment and therefore the likelihood of approval of clinical studies.

Certainly, increasing dosage would provide more reliable and longer block (and cause significant cardiovascular side effects). In rat pilot studies, we found that clinical neurotoxicity (as measured by neurobehavioral endpoints) begins at approximately 10% (n = 4, data not shown). Of note, for this pilot study, ephedrine HCl (Sigma, St. Louis, MO) was used, as the ephedrine sulfate used in all other experiments was identical to that used in the operating room and comes only at a concentration of 5% (50 mg in 1 ml). In humans, there are only limited data available regarding ephedrine's neurotoxicity when the drug is given systemically.²⁴ Interestingly, no neurologic sequelae were reported when ephedrine was given intrathecally or epidurally.^{6,7,9,18,25,26} However, subtle neurologic deficits may not have been reported.

This study has shown that at least some of the LA properties of ephedrine are due to the fact that it is a sodium channel blocker. Other contributions to the mechanism of peripheral nerve blockade may include inhibition of other ion channels or activation of novel subtypes of α_2 -adrenergic receptors similar to spinal cord.^{27,28} Traditionally, we have thought of ephedrine as an indirect sympathomimetic that may cause vasoconstriction, which delays LA reabsorption, thereby prolonging duration. Although this may still be part of the mechanism, other potent vasoconstrictors, such as phenylephrine, and even epinephrine and norepinephrine did not provide any block in the same model (n = 3-5 rats/drug, data not shown) up to dosages with se-

vere cardiovascular side effects. *In vitro* pilot studies revealed that epinephrine and norepinephrine had an estimated IC_{50} of approximately $1,600 \mu M$ for Na^+ current inhibition in the same cell line under identical conditions, although their cardiovascular potency is several times higher than that of ephedrine ($n = 3$ cells/drug, data not shown).

One of the goals of our laboratory is to decrease toxicity of clinical and investigational LAs by coinjection with synergistically acting drugs. A synergistic effect, one in which the combined effect of two drugs is greater than the sum of the effect of each drug given alone, allows dose reduction and side effect restriction while improving efficacy. However, in preliminary studies, when combining bupivacaine and ephedrine at equipotent dosages in human embryonal kidney cells expressing $Na_v1.4$, we found an IC_{50} of $3.1 \pm 0.1 \mu M$ at -60 mV for bupivacaine (for comparison, when bupivacaine was used without ephedrine, the IC_{50} was $7.9 \pm 0.2 \mu M$), suggesting a merely additive interaction ($n = 4$ cells, data not shown) and indicating that a potential synergistic interaction must be located beyond the channel protein. Of note, the IC_{50} of $3.1 \pm 0.1 \mu M$ at -60 mV for bupivacaine (when used in combination with ephedrine) in human embryonal kidney cells expressing $Na_v1.4$ was similar to that of $3.9 \pm 0.1 \mu M$ in GH_3 cells expressing $Na_v1.1$, 1.2 and 1.3 under identical conditions ($n = 4$ cells, data not shown).

In conclusion, because ephedrine has been shown to have LA properties *in vitro* and *in vivo* and a significantly longer sensory/nociceptive block when administered either alone or in combination with bupivacaine, future studies with a concentration of ephedrine not exceeding 1% to determine the presence or absence of synergism by formally constructing dose-response curves and isobolograms, as described,^{29,30} are justified.

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