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Structure–Activity Relation of N-alkyl Tetracaine Derivatives as Neurolytic Agents for Sciatic Nerve Lesions

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Background: **N-butyl tetracaine has local anesthetic and neurolytic properties. An injection of this drug at the rat sciatic notch produces rapid onset and nerve impairment lasting >1 week. This study aimed to elucidate the structure–activity relation of various tetracaine derivatives to design better neurolytic agents.**

Methods: **N-alkyl tetracaine salts (n = 2–6) were synthesized, and their ability to elicit sciatic nerve impairment of sensory and motor functions *in vivo* was tested in rats. A single dose (0.1 ml at 37 mM) was administered close to the sciatic nerve at the sciatic notch. Regeneration was assessed morphologically in transverse sections of treated nerves. Finally, the drug potency in blocking Na^+ currents was studied under voltage-clamp conditions.**

Results: **N-ethyl and N-propyl tetracaine derivatives were non-neurolytic and elicited complete sciatic nerve block lasting 3–7 h. In contrast, N-butyl, N-pentyl, and N-hexyl tetracaine derivatives were strong neurolytic agents and elicited functional impairment of sciatic nerve for >1 week. All derivatives were strong Na^+ channel blockers, more potent than tetracaine if applied intracellularly. External drug application showed marked differences in their wash-in rate: tetracaine > N-hexyl > N-butyl > N-ethyl tetracaine. All derivatives were trapped within the cytoplasm and showed little washout within 7 min.**

Conclusions: **When n-alkylation is 4–6, n-alkyl tetracaine appeared as a strong neurolytic agent. Neurolytic derivatives retained their local anesthetic activity and elicited rapid onset of nerve block after injection. Such derivatives are potential local anesthetic–neurolytic dual agents for chemical lesions of the sciatic nerve. (Key words: Local anesthetic; nerve regeneration; sodium channel blocker.)**

NEUROLYTIC agents such as phenol (5–10%) and absolute alcohol have been used to alleviate chronic and intractable cancer pain for several decades.[1] This type of treatment has retained its clinical utility and may be the preferred method for some well-defined cases. Phenol and absolute alcohol are highly destructive to tissues but both have weak or no local anesthetic activity *in vivo*.[1-3] Recently we described a novel local anesthetic/neurolytic dual agent, N-butyl tetracaine, which has rapid onset and complete ultralong sciatic nerve impairment that can last several weeks.[4] N-butyl tetracaine also blocks Na^+ currents tonically when the nerve is stimulated infrequently. The potency of externally applied N-butyl tetracaine in Na^+ current block is comparable to that of its parent drug, tetracaine, which is a potent local anesthetic. When the nerve is stimulated at 2 Hz, N-butyl tetracaine causes profound use-dependent inhibition of Na^+ currents, as most local anesthetics do.

In addition to its local anesthetic properties, N-butyl tetracaine causes severe nerve degeneration, as detected 3–5 weeks after a single injection of 0.1 ml at 37 mM close to the sciatic nerve of the rat at the sciatic notch.[4] Thus the prolonged functional impairment by N-butyl tetracaine is due to the sciatic nerve lesion and not due to continued local anesthetic block. As a result, functional impairment of the sciatic nerve may be caused by local anesthetic activity of the drug (such as the rapid onset of block) or caused by neurolytic activity of the drug (such as the prolonged impairment of sciatic

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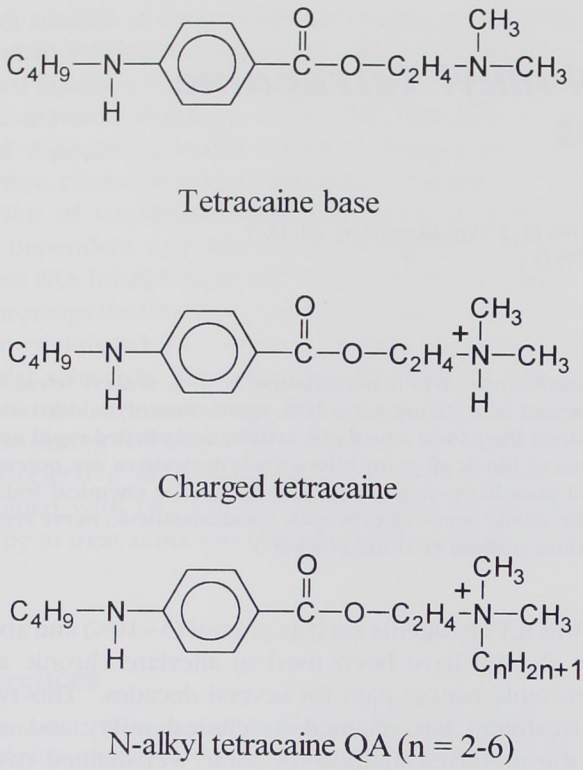

Fig. 1. Chemical structures of tetracaine and its quaternary ammonium derivatives.

nerve function by a chemical lesion). Nerve regeneration may occur after nerve degeneration, because sciatic nerve functions recover 9 weeks after the injection. The underlying mechanism for the neurolysis caused by N-butyl tetracaine is not known. This information will be important for the future design of neurolytic agents better than phenol and absolute alcohol. As a first step toward this goal, we began to examine the structure-activity relation of various N-alkyl tetracaine derivatives.

Materials and Methods

Organic Synthesis of N-alkyl Tetracaine Quaternary Ammonium Derivatives

Quaternary ammonium compounds of N-alkyl tetracaine (fig. 1), in which the alkyl addition is an n-alkyl group of two to six carbon atoms, were synthesized from bromoalkanes according to the method described by Wang *et al.*[5] For alkyl groups of two to five carbon atoms, a 1:2 molar ratio of tetracaine base (Sigma Chemical Co., St. Louis, MO) and bromoalkane (Aldrich, Milwaukee, WI) was used. For N-hexyl tetracaine, the molar ratio of the starting materials was 1:1. The mixture in absolute ethanol was refluxed for about 30 h, and the reaction was followed by normal-phase thin-layer chromatography (Fisher Scientific, Pittsburgh, PA), developed with chloroform/ethanol (9:1, vol:vol) or with 96% ethanol/0.8 M NH_4Cl (4:1, vol:vol), and visualized in iodine vapor or by ultraviolet light. The solvent was evaporated when the reaction was complete, and the product was washed several times with warm hexane and then with ethyl ether. N-hexyl tetracaine was dissolved in ethanol and reprecipitated by ethyl ether. The other products were purified by repeated recrystallization from ethanol or from appropriate mixtures of hot ethanol and ethyl ether. The products were dried in a vacuum. The yields were approximately 55%. Products were >97% pure as judged by thin-layer chromatography systems. Structural analyses of the alkyl-tetracaine quaternary ammoniums by mass spectrometry yielded molecular masses of 293.1, 307.2, 321.4, 335.1, and 349.3 daltons, which are consistent with the structures of the corresponding quaternary ammonium ions (from ethyl to hexyl; see fig. 1). Bromide was the counter ion for the quaternary ammonium compounds.

Sensory and Motor Block of Rat Sciatic Nerves

Neurologic evaluations of rat sciatic nerve impairment were conducted according to methods described previously.[4,6] These protocols were approved by the Harvard Medical Area Standing Committee on Animals. Drugs were injected and all sciatic nerve functions were examined by the same investigator (M.V.) to avoid possible differences in behavioral evaluation. Drugs at a final concentration of 37 mM in isotonic saline (equivalent to a 1.11% tetracaine-hydrochloric acid concentration) were coded and injected in a volume of 0.1 ml close to the sciatic nerve at the sciatic notch. Functional impairment was assayed by comparing values before and at various intervals after injection. Changes of function were estimated and normalized as percentages of maximal possible effect. Complete impairment of function was defined as 100% maximal possible effect and no change of function as 0% maximal possible effect. This definition does not imply that the functional assays used a continuous variable but were applied to normalize the data for comparison. Detailed descriptions of rat

handling and subsequent behavior tests can be found in Thalhammer *et al.*[6] and are summarized briefly here.

Proprioception was evaluated according to combined postural reactions (such as "hopping" and "tactile placing," described subsequently) and was scored from 3 (normal postural reactions) to 0 (complete lack of postural reactions); a score of 0 represented 100% maximal possible effect. To evaluate tactile placing, the toes of one foot were ventroflexed and the dorsal surface of the paw was positioned onto the supporting surface while the animal was kept in normal resting posture. The ability to reposition the toes was evaluated (3 = normal, 2 = slightly impaired, 1 = severely impaired, 0 = absent). To assess hopping, the front half of the animal was lifted off the supporting surface so that the body's weight was supported by the hind limbs only. One hind limb at a time was then lifted and the animal's body was moved laterally. The ability of the animal to follow the lateral movement of the body by hopping with the weight-supporting limb was evaluated (3 = normal, 2 = slightly impaired, 1 = severely impaired, 0 = absent).

Motor function of the hind limbs was evaluated by the "extensor postural thrust." The rat was held upright so that hind limbs were extended and the body's weight was supported by the distal metatarsus. The force (measured in grams) necessary to bring the heel into contact with the platform of a balance was measured. The reduction in extensor thrust resulting from reduced extensor muscle tone was considered to represent reduced motor function; an absence of extensor postural thrust was defined as complete (100%) motor block.

Nociception was evaluated by measuring the latency of the withdrawal response to noxious pinch. Care was taken to avoid tissue injury resulting in hyperalgesia by properly spacing in time the stimulations. The fifth toe was pinched (up to 300 g) with a force-calibrated serrated forceps for 2 s, and the withdrawal response was assessed as 4 (normal, brisk, attempt to escape; withdrawal of the stimulated hind limb; attempts to bite the forceps; and vocalization); 3 (the same as 4, but slower than on the control side); 2 (the same as 3, but with one of the responses lacking); 1 (only a weak attempt to withdraw); and 0 (no response).

Morphologic Assay of Sciatic Nerve Regeneration after N-alkyl Tetracaine Injection

Rats with functional recovery of nociception after n-alkyl tetracaine injection were used for histologic studies (performed by D.C.A.). Some degrees of impairment of motor function and proprioception persisted in these rats when they were killed by pentobarbital overdose. The sciatic nerve was dissected from both legs, with the nerve contralateral to the injection site serving as a control. Excess adipose tissue was trimmed and the nerves were rinsed with saline solution. A suture was placed at the site of injection and each nerve sample was placed in a separate vial containing 4% glutaraldehyde in 0.1 M cacodylate buffer (Poly Scientific Research & Development Corp., Bay Shore, NY). Histologic cross-sectioning of resin (epon)-embedded nerve preparation was performed distal to the injection site (5–6 mm), as described by Anthony *et al.*[7] Results were recorded as normal or the extent of abnormality was noted. The number of degenerating fibers were calculated and the maximal thickness of the recovery zone was measured. Because the number of animals was not sufficient to make clear conclusions about these numbers, we used the semi-quantitative estimates of "abundant" axonal degeneration and the Fisher exact test to determine the statistical significance of morphologic changes.

Voltage Clamp Experiments in GH_3 Cells

Rat clonal pituitary GH_3 cells were purchased from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone Labs, Logan, UT) in a 5% carbon dioxide incubation chamber at 37°C. For Na^+ current recording, GH_3 cells were replated in a 35-mm culture dish, which was then used as a recording chamber. The standard whole-cell variant of the patch-clamp method was used for current recording.[8] The external solution contained 150 mM choline Cl, 0.2 mM $CdCl_2$, 2 mM $CaCl_2$, and 10 mM HEPES adjusted to *p*H 7.4 with tetraethylammonium hydroxide. Micropipettes were fabricated with a tip resistance of about 1 MΩ when filled with a Na^+ solution containing 100 mM NaF, 30 mM NaCl, 10 mM EGTA, and 10 mM HEPES adjusted to *p*H 7.2 with CsOH. After the patch membrane ruptured, the cell was allowed to equilibrate with the pipette solution for at least 15 min at a holding potential of −100 mV. N-alkyl tetracaine derivatives were applied externally to cells with a flow rate of about 0.12 ml/min through a series of narrow-bored capillary tubes positioned within 200 μm of the cell. Washout of drugs was performed with a tube containing the external solution without drug. In some

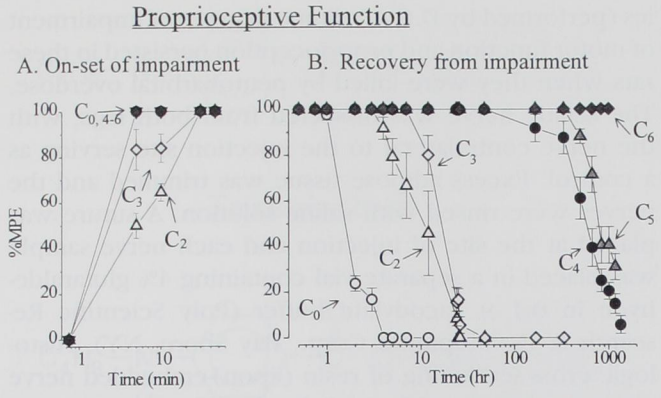

Fig. 2. Proprioceptive block of rat sciatic nerve by N-alkyl tetracaine derivatives. On-set (*A*) and recovery (*B*) time courses of proprioceptive impairment are expressed as a percentage of maximal possible effect (means ± SEM) after injection of 0.1 ml drug at 37 mM. Symbols indicate tetracaine (C_0, ○; n = 8), N-ethyl tetracaine (C_2, Δ; n = 8), N-propyl tetracaine (C_3, ◇ n = 14), N-butyl tetracaine (C_4 tetracaine, ●; n = 14), N-pentyl tetracaine (C_5, ▲; n = 7), and N-hexyl tetracaine (C_6 tetracaine, ♦; n = 9). Test intervals for *A* were before the injection and 5, 10, 30, and 45 min after the injection. Test intervals for *B* were 0.5, 0.75, 1, 2, 3, 4, 5, 7, 12, 24–26, 48, 168, 336, 504, 672, 840, 1,008, 1,176, and 1,344 h (1 wk = 168 h).

cases, drugs were applied intracellularly by gravity through an internal perfusion pipette holder (Adams & List Associates, Westbury, NY) into which a small quartz tube filled with the drug-containing internal solution was inserted.[9] Control Na^+ currents were first recorded in normal internal solution and the internal perfusion of drug was then followed. Under these conditions, the block of Na^+ currents usually took at least 15–20 min to reach steady state due to the small pipette tip compared with the cell volume. Voltage clamp protocols were created using pClamp software (Axon Instruments, Foster City, CA). Leak and capacitance were subtracted using the patch-clamp device (EPC-7; List-Electronic, Dramstadt/Eberstadt, Germany) and by pClamp software (except for the use-dependent block protocol). All experiments were performed at room temperature (23 ± 2°C).

Statistical Analysis

A Mann-Whitney rank sum test or a Kruskal-Wallis one-way analysis of variance on ranks (Sigmastat; Jandel Scientific Software, San Rafael, CA) was used to assess the significance of differences in the magnitude and duration of functional changes detected during neurologic evaluation after the injection of tetracaine and N-alkyl tetracaine derivatives. An unpaired Student's *t* test (Sigmastat) was used to evaluate the significance of drug-induced changes in the rate and the steady state of tonic and use-dependent block. A Fisher exact test was used to measure the statistical significance of morphologic changes. A probability value <0.05 was considered significant.

Results

Two Distinct Onset Time Courses of the Sciatic Nerve Impairment by N-alkyl Tetracaine Derivatives

The time courses of proprioceptive, motor, and nociceptive impairment induced by various quaternary derivatives of N-alkyl tetracaine are shown in figures 2A, 3A, and 4A, respectively. After a single drug injection of 0.1 ml at 37 mM at the rat sciatic notch, the onset times were much longer for N-ethyl and N-propyl tetracaine than for N-butyl, N-pentyl, and N-hexyl tetracaine. For N-ethyl and N-propyl tetracaine compounds, it took about 30 min for the block to develop fully (figs. 2A–4A), whereas for N-butyl, N-pentyl, and N-hexyl tetracaine compounds, it took about 5 min or less (figs. 2A–4A). The differences in the time to achieve complete block between these two groups of drugs are significant ($P < 0.05$). Injection of N-ethyl tetracaine with precision was important for eliciting sciatic nerve block; in

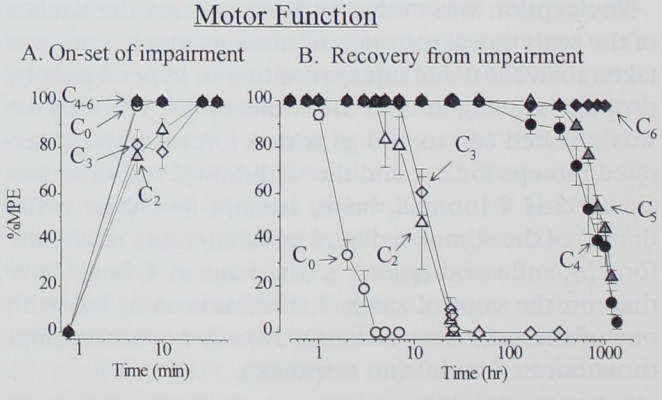

Fig. 3. Motor block of rat sciatic nerve by N-alkyl tetracaine derivatives. On-set (*A*) and recovery (*B*) time courses of motor impairment are expressed as a percentage of maximal possible effect (means ± SEM) after injection of 0.1 ml drug at 37 mM. Symbols, sample numbers, and test intervals are the same as in figure 2.

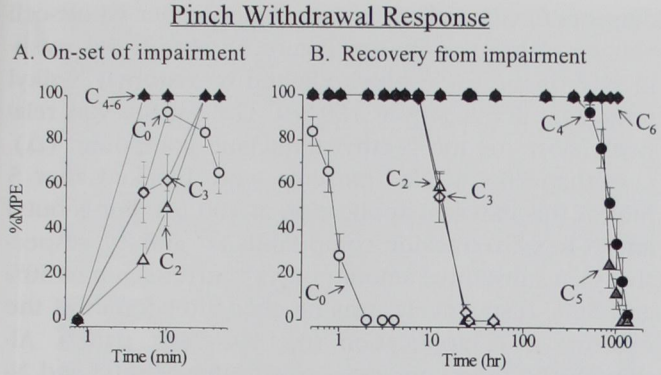

Fig. 4. Nociceptive block of rat sciatic nerve by N-alkyl tetracaine derivatives. On-set (*A*) and recovery (*B*) time courses of nociceptive impairment are expressed as a percentage of maximal possible effect (means ± SEM) after injection of 0.1 ml drug at 37 mM. Symbols, sample numbers, and test intervals are the same as in figure 2.

4 of 12 rats, imprecise injection of N-ethyl tetracaine failed to produce sciatic nerve block. For comparison, other derivatives of tetracaine had a success rate of >90%. The rats that did not respond to drugs received repeated injections in the other leg the next day. This result indicates that there is an apparent cut-off phenomenon for fast onset of N-alkyl tetracaine; compounds with an alkyl chain of 4 or longer produce block faster than do those with an alkyl chain of 3 or less. The onset of tetracaine block for proprioceptive and motor functions was fast and comparable to that of N-butyl, N-pentyl, and N-hexyl tetracaine (figs. 2A, 3A). The onset of tetracaine block for nociception was, however, slower and did not reach complete block in all rats (fig. 4A).

Long and Ultralong Duration of Complete Sciatic Nerve Impairment by N-alkyl Tetracaine

The duration of complete sciatic nerve block for proprioception, motor function, and response to noxious pinch by N-ethyl and N-propyl tetracaine lasted for 3-7 h (figs. 2B-4B). This duration of block was significantly longer than that of the parent drug, tetracaine, which induced complete block of proprioception and motor function for 45-60 min (figs. 2B-3B; $P < 0.05$); nociception was not completely blocked in all rats by tetracaine (fig. 4B). In contrast, the duration of complete sciatic nerve impairment by N-butyl, N-pentyl, and N-hexyl tetracaine was significantly longer than that by N-ethyl and N-propyl tetracaine ($P < 0.05$). These compounds induced ultralong impairment of sciatic nerve function for 1-6 weeks (168-1,008 h; figs. 2B-4B). Injection of N-hexyl tetracaine led to injury of the lateral part of the tarsus in all eight rats after 1-6 weeks. It is not clear whether this injury was associated with neuropathic pain or was a result of grooming with absence of sensory feedback. We have not tried to measure directly the presence of neuropathic pain. There was, however, no indication of discomfort in the appearance of these animals, their body weight, or their behavior. All rats injected with N-hexyl tetracaine were killed at 6 weeks because of inflammation and subsequent tarsus lesions.

Two Distinct Recovery Time Courses of Functional Impairment

With a single injection of 0.1 ml 37 mM N-alkyl tetracaine derivatives, impaired sciatic nerve functions recovered with two distinct time courses. For N-ethyl and N-propyl tetracaine, impaired functions recovered completely within 36 h (figs. 2B-4B). In contrast, for N-butyl, N-pentyl, and N-hexyl tetracaine, impaired functions recovered slowly. Within this group, rats injected with N-butyl-tetracaine started to recover in 1-7 weeks (168-1,176 h) and reached complete recovery at about 8 weeks (1,344 h), which was significantly slower than 36 h for N-ethyl and N-propyl tetracaine (figs. 2B-4B; $P < 0.05$). Rats injected with N-pentyl tetracaine began to recover in 2-6 weeks. Nociceptive responses reached complete recovery at about 6-7 weeks, but some deficits in proprioception and motor function remained at 7-8 weeks for rats injected with N-pentyl tetracaine. Because prolonged impairment of sciatic nerve function for several weeks often leads to muscle atrophy, routine muscle exercise may be needed to improve the recovery of proprioception and motor function for rats treated with N-pentyl tetracaine. The recovery of proprioception and motor function appeared with similar time courses in rats treated with N-pentyl tetracaine and N-butyl tetracaine; the differences in these data points are not significant. The functional impairment of nociception, proprioception, and motor function in rats injected with N-hexyl tetracaine did not recover significantly up to week 6. As dictated by our institution-approved animal protocol, these rats were killed by the sixth week because of inflammation and subsequent lesion of their tarsus.

Neurolytic versus *Non-neurolytic Effects of N-alkyl Tetracaine Derivatives on Sciatic Nerve Fibers In Vivo*

We previously showed[4] that severe nerve degeneration occurred 3–5 weeks after a single injection of N-butyl tetracaine at 37 mM. In this study, we allowed recovery of nociceptive function after the injection of N-propyl and N-pentyl tetracaine compounds. These rats with proper nociceptive function were subsequently killed, and rat sciatic nerve samples from the injected and the contralateral sides were excised, fixed, and sectioned. We noted that some deficits in proprioception and motor function persisted in rats treated with N-pentyl tetracaine (figs. 2B–4B). By light microscopy of epon-embedded sections of nerve, control specimens showed the normal axonal population of the sciatic nerve (fig. 5A, upper left panel; total number of animals examined, n = 9). The findings in sections 14 days after a single injection of N-propyl tetracaine at 37 mM were similar, with a completely normal axonal population (fig. 5B, upper right panel; n = 5). The absence of any evidence of degeneration at 14 days indicates that N-propyl tetracaine does not possess the same neurolytic properties of the related N-butyl and N-pentyl tetracaine. In contrast, there was abundant axonal degeneration in rats 48 days (fig. 4C, middle left panel; n = 5; $P < 0.05$ by the Fisher exact test) or 68 days (fig. 5D, middle right panel; n = 5; $P < 0.05$) after a single injection of N-pentyl tetracaine at 37 mM. In addition, regions of small-caliber myelinated axons typical of axonal regeneration were abundant 68 days after N-pentyl tetracaine injection (fig. 5D, middle right panel; n = 5; $P < 0.05$). These regenerative fibers were especially prominent toward the periphery of individual fascicles and were clustered in these areas (fig. 5F, bottom right panel) compared with control fascicles (fig. 5E, bottom left panel).

Tonic Block of Na^+ Channels by N-alkyl Tetracaine Derivatives

Typical tertiary-amine LAs, such as tetracaine, inhibit voltage-gated Na^+ channels rapidly within a few minutes after external application (fig. 6A, left panel). This is not usually the case for quaternary derivatives. For example, QX-314, a lidocaine derivative (*i.e.*, N-ethyl lidocaine) is essentially inactive to neuronal Na^+ channels when applied externally at 1 mM because it fails to penetrate the cell membrane effectively.[10] Therefore the ability of N-alkyl tetracaine derivatives to block Na^+ currents in GH_3 cells was determined under whole-cell voltage clamp conditions. Figure 6B shows the washin rate of the tonic block elicited by external N-alkyl tetracaine derivatives at 100 μM. The washin was relatively slow for the N-ethyl tetracaine compound (Δ). Less than 20% of Na^+ currents were blocked after 5 min of this external application at 100 μM. For N-butyl and N-hexyl tetracaine compounds (∇ and $\diamond$, respectively), a substantial amount of Na^+ currents, up to 50% and 80%, respectively, was blocked after 5 min of the external drug application (fig. 6A, right panel). Although the time courses of washin for N-butyl and N-hexyl tetracaine were faster than for N-ethyl tetracaine, they were still much slower than the tertiary-amine parent drug tetracaine ($\bigcirc$). These results are consistent with the *in vivo* block by N-alkyl tetracaine derivatives; for example, the onset of sciatic nerve block by N-ethyl tetracaine is much slower than that by N-butyl-tetracaine after a single injection (figs. 2A–4A).

After N-alkyl tetracaine derivatives entered the cell, the external drugs were washed out and the cells were superfused continuously with drug-free external solution. Figure 7 shows the recovery time courses of the tonic block during the external washout of N-alkyl tetracaine derivatives. None of these derivatives appeared to be washed out from the cells quickly because the tonic block remained essentially constant after 7 min of external superfusion. Prolonged superfusion of cells for 35 min slowly reversed the block by N-butyl-tetracaine,[4] but the time course was relatively slow and the recovery remained incomplete. In contrast, the tertiary-amine parent drug, tetracaine ($\bigcirc$), could be removed readily from the cells; within 2 min of washout, Na^+ currents recovered to the level before drug application. This result indicates that N-alkyl tetracaine derivatives with a permanent positive charge are trapped within the cell after they successfully penetrate the cellular membrane.

For the true blocking potency of N-alkyl tetracaine derivatives on Na^+ channels, it was necessary to apply drugs directly to the inside of the cells, thus bypassing the membrane barrier for the permanently charged compounds. Figure 8A (right panel) shows that at 50 μM, internally perfused N-hexyl tetracaine was extremely potent in blocking the Na^+ currents in GH_3 cells. N-ethyl, N-butyl, and N-hexyl tetracaine blocked more than 90% of the Na^+ currents at 50 μM internal concentration (fig. 8B). The potency of these derivatives is significantly higher than their tertiary-amine par-

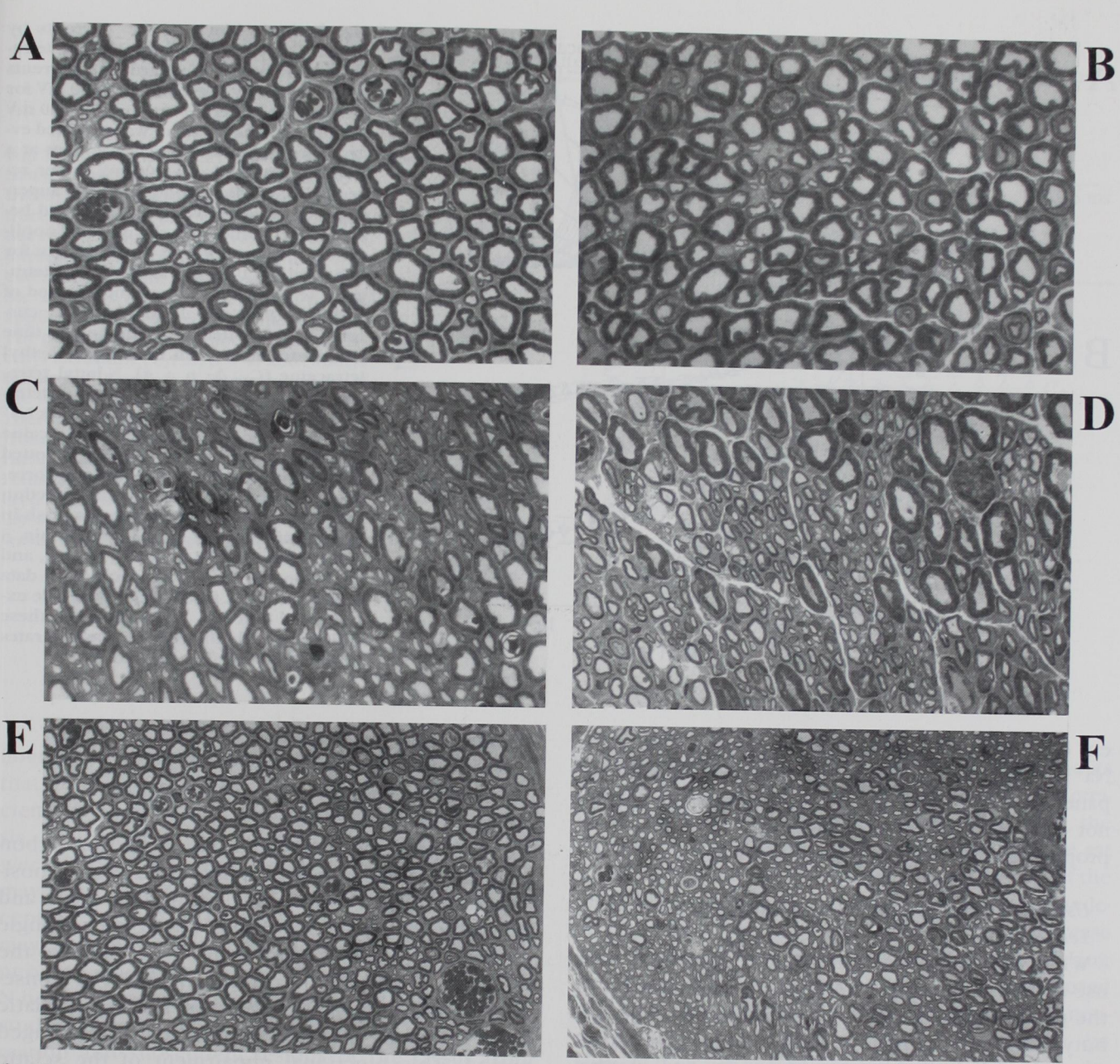

Fig. 5. Regeneration of rat sciatic nerve after neurolytic N-pentyl tetracaine injection. (*A*) Control sciatic nerve (toluidine blue stain; magnification, ×520). (*B*) Sciatic nerve 14 days after injection of N-propyl tetracaine (37 mM; toluidine blue stain; magnification, ×520). (*C*) Sciatic nerve 48 days after injection of N-pentyl tetracaine (37 mM). Some degenerating fibers and small-caliber (regenerating) fibers are present (toluidine blue stain; magnification, ×520). (*D*) Sciatic nerve 68 days after injection of N-pentyl tetracaine (37 mM). Degenerating fibers are fewer than at 48 days, and small-caliber (regenerating) fibers are more abundant (toluidine blue stain; magnification, ×520). (*E*) Control sciatic nerve. The overall axonal population is very uniform (toluidine blue stain; magnification, ×260). (*F*) Sciatic nerve 68 days after injection of N-pentyl tetracaine (37 mM). There is a tendency for the small-caliber (regenerating) fibers to be most abundant adjacent to the perineurium (toluidine blue stain; magnification, ×260).

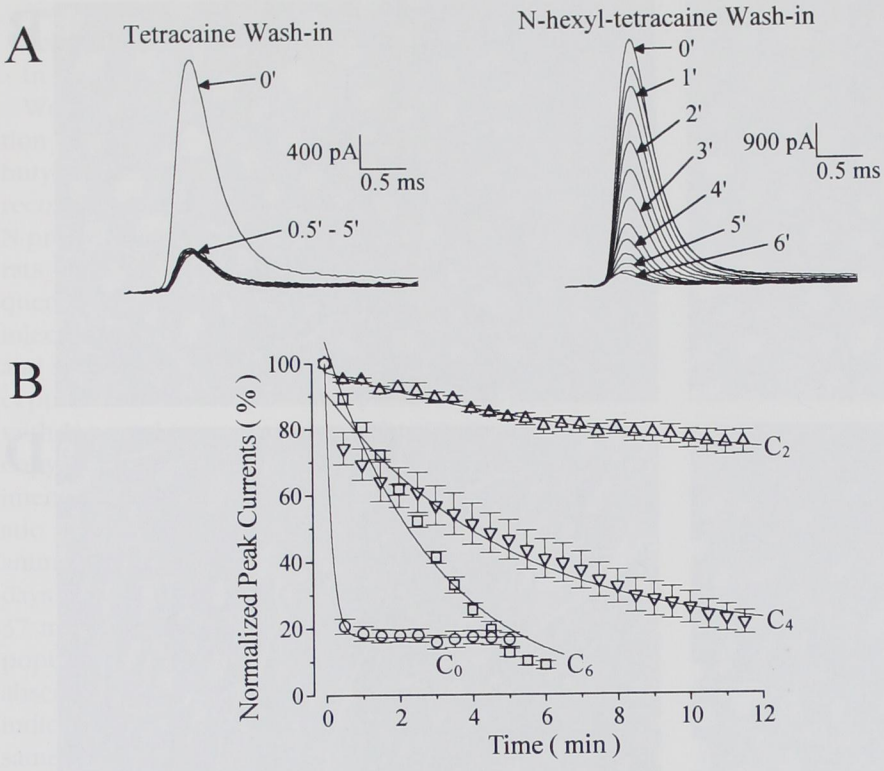

Fig. 6. Tonic block of Na^+ currents by washin of tetracaine and N-alkyl tetracaine derivatives. Outward Na^+ currents were elicited by a test pulse of +30 mV for 5 ms applied after a prepulse of −130 mV for 100 ms. The protocol was repeated every 0.5 min while the cell was kept at a holding potential of −100 mV under reversal Na^+ gradient conditions. (*A*) Superimposed consecutive traces recorded before (time 0) and after the external application of tetracaine (left panel; 100 μM for a period of 0.5′–6′) and N-hexyl-tetracaine (right panel; 100 μM for a period of 0.5′–6′). (*B*) Normalized peak Na^+ currents were plotted as a function of time after external washin of 100 μM N-ethyl tetracaine (C_2, Δ; n = 4), N-butyl tetracaine (C_4, ▽; n = 5), and N-hexyl tetracaine (C_6, □; n = 5). Normalized peak Na^+ currents after external 100 μM tetracaine washin (C_0, ○; n = 4) were also plotted for comparison. Each data set was force-fitted by a single exponential function (solid line) to extract the relative wash-in rate (τ) of 9.9 ± 3.3 min, 4.9 ± 0.9 min, 3 ± 0.1, and 0.16 ± 0.03 for C_2, C_4, C_6, and C_0, respectively. Although some data points were not fitted well by a single exponential function, for comparison these fittings provide the relative wash-in rates of N-alkyl tetracaine derivatives.

ent drug tetracaine, which blocked about 50% of the Na^+ currents at the same concentration (fig. 8A, left panel; $P < 0.05$). Thus N-alkyl tetracaine derivatives not only retain but also enhance the local anesthetic properties of their parent drug tetracaine.

Use-dependent Block of Na^+ Channels by N-alkyl Tetracaine

When GH_3 cells were stimulated repetitively, clear use-dependent block of Na^+ currents occurred in the presence of externally applied tetracaine and N-butyl tetracaine.[4] Similarly, N-alkyl tetracaine derivatives also elicited this type of use-dependent phenomenon when applied internally. Figure 9 shows that repetitive pulses significantly reduced peak Na^+ current amplitude. The time courses of use-dependent block elicited by internal N-ethyl and N-butyl tetracaine compounds were similar to those elicited by external application of N-butyl tetracaine.[4] Thus our results show that N-alkyl tetracaine derivatives even at low concentrations can produce use-dependent block of Na^+ currents in a manner similar to their parent drug tetracaine.

Discussion

Neurolytic versus Non-neurolytic N-alkyl Tetracaine Derivatives

This report shows that alkylation of the carbon chain of tetracaine by 4–6 at the tertiary amine position renders the compounds highly neurolytic and can cause severe axonal degeneration after a single injection of these derivatives at 37 mM close to the sciatic nerve at the rat sciatic notch. The consequence of the axonal degeneration is that the sciatic nerve functions will remain impaired for a prolonged period. This functional impairment of the sciatic nerve by neurolytic N-alkyl tetracaine can last weeks or months (figs. 2B–4B), but the sensory and motor functions may return when the nerve fibers regenerate. Regeneration could take many weeks and probably depends on the drug injected and on the distance between the site of injection and the distal innervation site at the nerve endings.[11] Regeneration is evident under the light microscopic examinations of sciatic nerve preparations treated with N-pentyl tetracaine (fig. 5). It is interesting to note that some

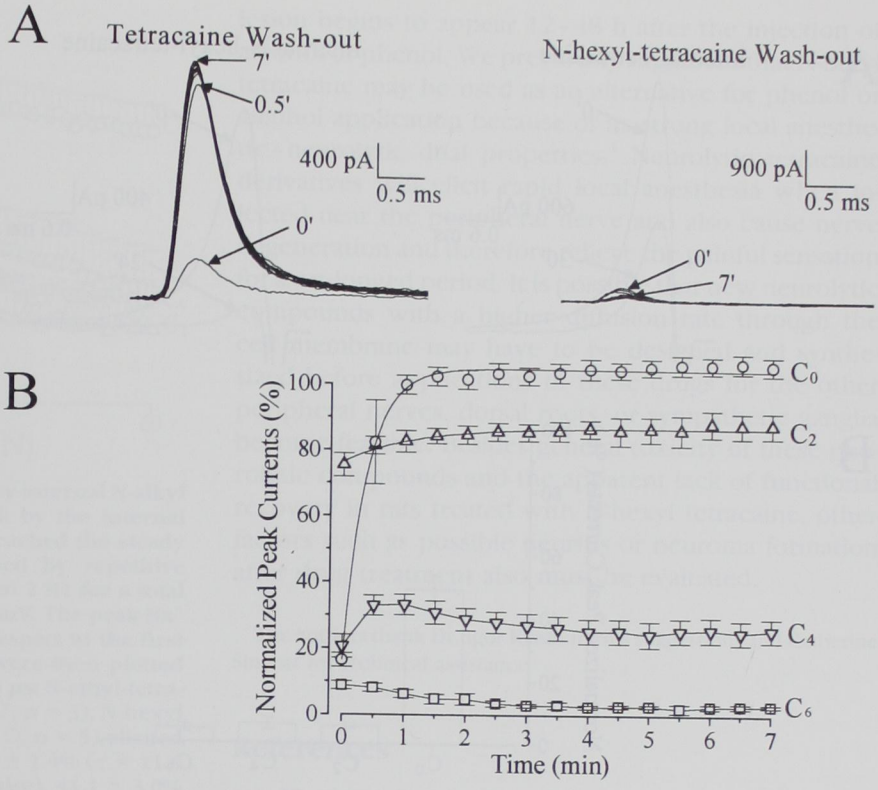

Fig. 7. Tonic block after external washout of tetracaine and N-alkyl tetracaine derivatives. (*A*) Superimposed consecutive traces recorded every 0.5 min with washout of tetracaine (*left panel*) and N-hexyl tetracaine (*right panel*) with a drug-free external solution. (*B*) Normalized peak Na^+ currents were plotted against time at application of drug-free external solution. The peak Na^+ currents blocked by both N-ethyl tetracaine (C_2, Δ; n = 4) and N-butyl tetracaine (C_4, ∇; n = 5) recovered from the tonic block slowly and incompletely. There was little recovery of Na^+ currents from cells treated with 100 μM N-hexyl tetracaine (C_6, $\square$; n = 5). In contrast, the currents blocked by tetracaine (C_0, $\bigcirc$) recovered completely within 2 min (101.7 $\pm$ 2.9% with a τ = 0.37 $\pm$ 0.06 s; n = 4) after washout with drug-free external solution.

fibers are spared from degeneration, which suggests that neurolytic compounds may fail to reach a sufficient concentration in these fibers to cause neurolysis. A number of fibers also fail to regenerate by weeks 7–9 after N-pentyl tetracaine injection, which may contribute to the continued deficits in proprioception and motor function. Longer recovery time may be required for the structure of the rat sciatic nerve to regenerate completely. In rats treated with N-hexyl tetracaine, nerve regeneration cannot be studied until we find a protocol to prevent inflammation and subsequent lesions of the tarsus from developing in the animals.

Probable Cause for the Sciatic Nerve Lesion by Neurolytic N-alkyl Tetracaine Derivatives

Tetracaine and its derivatives with alkylation of 3 or fewer do not possess neurolytic activity toward sciatic nerve. In comparison, tetracaine derivatives with alkylation of 4–6 possess neurolytic activity *in vivo* after a single injection at 37 mM. This apparent difference among tetracaine derivatives in neurolytic activity may be understood if we consider the drug diffusion and its *in vivo* bioavailability after a single injection of tetracaine at the rat sciatic notch.[12] At the initial stage, the intraneural concentration of tetracaine within the rat sciatic nerve will rapidly increase to about 1–2% of the injected concentration and then decrease to an undetectable level within 70 min.[12] This transient increase and decrease of intraneural tetracaine may not be long enough or the concentration high enough to damage the nerve fibers irreversibly. However, tetracaine is neurotoxic and cause nerve damage *in vivo* when extremely high concentrations are applied.[13] To cause local neurolysis of the sciatic nerve, we propose that these compounds must cross the cell membrane and enter the cytoplasm. Non-neurolytic N-alkyl tetracaine derivatives ($n \leq 3$) cannot cross the cell membrane readily, as indicated by their slow on-rate action in sciatic nerve block. The neurotoxicity of high concentration tetracaine and the neurolysis of neurolytic N-alkyl tetracaine may be due to a common mechanism. The molecular mechanism of neurolysis induced by N-alkyl tetracaine remains unresolved.

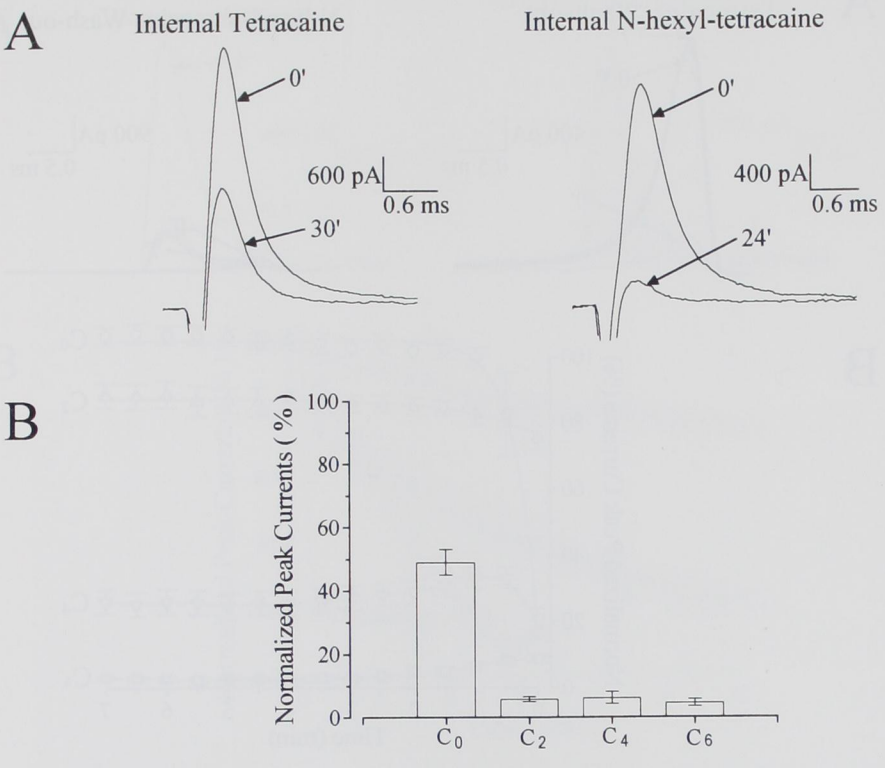

Fig. 8. Tonic block of Na^+ currents by internal perfusion of 50 μM N-alkyl tetracaine derivatives. (*A*) Superimposed current traces were recorded before and after internal perfusion of 50 μM tetracaine (*left panel*) and 50 μM N-hexyl tetracaine (*right panel*). The block reached steady-state values at 30 min and 24 min, respectively. (*B*) Tonic block of Na^+ currents by internal 50 μM N-alkyl tetracaine derivatives is compared with means ± SEM. With internal application of N-ethyl tetracaine, the peak Na^+ current amplitudes were on average 5.8 ± 0.8% (C_2, n = 6) of the untreated control; with N-butyl tetracaine, the average was 6.2 ± 1.9% (C_4, n = 6); and with N-hexyl tetracaine, the average was 4.6 ± 1.2% (C_6, n = 6). In contrast, with 50 μM tetracaine applied internally, the peak Na^+ current amplitudes were on average 49 ± 4% (C_0, n = 9). The difference between N-alkyl tetracaine derivatives and tetracaine was significant ($P < 0.05$). Among N-alkyl tetracaine derivatives, the differences in tonic block were not significant. The intracellular perfusion was achieved by the gravity force and the internal solution was perfused at a constant rate of 15 μl/min. Time required to observe macroscopic current change was estimated to be 7–10 min after the perfusion began. New steady-state block could be reached within 20–40 min.

Local Anesthetic Properties of N-alkyl Tetracaine Derivatives

All N-alkyl tetracaine derivatives are more potent than tetracaine in their local anesthetic activities when applied internally. By internal application, quaternary ammonium compounds bypass the barrier of the cell membrane, thus permitting direct measurement of their potency. We found that tetracaine alkylated with two to six carbon atoms blocked the Na^+ current by >90% when applied internally at 50 μM, whereas tetracaine blocked about 50% (fig. 8B). This finding is consistent with a previous report showing an additional hydrophobic binding domain within the local anesthetic binding site.[14] Therefore, the presence of an alkyl moiety on tetracaine increases its potency significantly.

In contrast, external application of these permanently charged compounds shows significant differences in their wash-in kinetics as well as their apparent potency in blocking the Na^+ current. The sequence of the wash-in kinetics of the four tested compounds is as follows: tetracaine > N-hexyl tetracaine > N-butyl tetracaine > N-ethyl tetracaine. Based on these results, we conclude that the higher the alkylation number (n = 2–6), the faster the washin of external N-alkyl tetracaine. After 10 min of drug superfusion, N-ethyl, N-butyl, N-hexyl tetracaine, and tetracaine at 100 μM block about 20%, 74%, 95%, and 80% of Na^+ current, respectively. Clearly, the membrane barrier plays a significant role in determining the potency of these quaternary derivatives of tetracaine *in vitro* as well as *in vivo*. N-ethyl tetracaine has the slowest wash-in rate and N-hexyl tetracaine the fastest for the N-alkyl tetracaine derivatives. However, the externally applied parent drug tetracaine is still faster than the externally applied N-hexyl tetracaine compound in blocking Na^+ current. This is because tetracaine, a tertiary amine local anesthetic, can be in a neutral (unprotonated) form (see fig. 1) that penetrates the cell membrane readily.[15] Despite the marked differences in wash-in kinetics, all N-alkyl tetracaine derivatives are slow to washout, which suggests that these compounds are easily trapped within the cytoplasm. The trapped N-alkyl tetracaine derivatives can also cause use-dependent block of Na^+ current in a manner similar to traditional local anesthetics. This

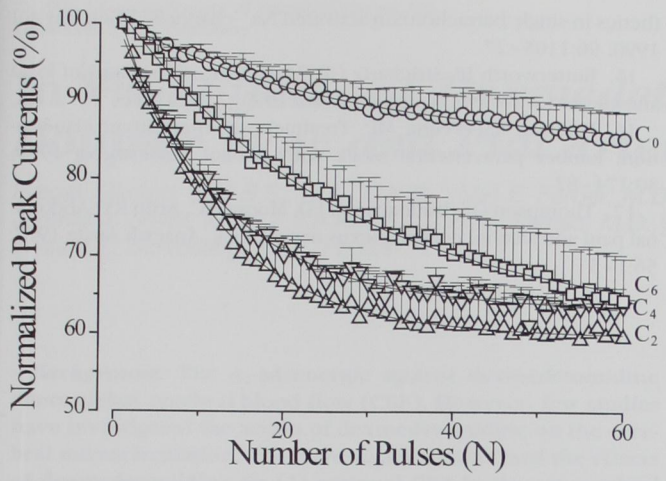

Fig. 9. Use-dependent block of Na^+ currents by internal N-alkyl tetracaine derivatives. After the tonic block by the internal application of N-alkyl-tetracaine at 10 μM reached the steady state, use-dependent inhibition was elicited by repetitive pulses of +30 mV for 24 ms at a frequency of 2 Hz for a total of 60 pulses while the cell was held at −100 mV. The peak Na^+ current amplitudes were normalized with respect to the first pulse of the set. The normalized currents were then plotted against the pulse number. At steady state, 10 μM N-ethyl-tetracaine (C_2, Δ, n = 6), N-butyl-tetracaine (C_4, ∇, n = 3), N-hexyl tetracaine (C_6, $\square$, n = 7), and tetracaine (C_0, $\bigcirc$, n = 5) elicited use-dependent block of Na^+ current by 43.1 ± 1.4% (τ = 11.4 ± 1.3 pulse), 37.5 ± 0.4% (τ = 12.4 ± 0.2 pulse), 41.1 ± 3.0% (τ = 26.3 ± 4.8 pulse), and 16.2 ± 1.7% (τ = 26.7 ± 6.7 pulse), respectively. The magnitudes of the use-dependent block at the sixtieth pulse for C_2, C_4, and C_6 are significantly greater than for C_0 ($P < 0.05$). The τ values were single exponential fits of each data set (*solid lines*).

result explains why a single injection of N-ethyl and N-propyl tetracaine at 37 mM results in slower onset of *in vivo* sciatic nerve block than a single injection of N-butyl, N-pentyl, N-hexyl tetracaine, and tetracaine (figs. 2A-4A).

Possible Applications of Local Anesthetic-Neurolytic Dual Agents as Therapeutic Drugs

Traditional neurolytic agents such as phenol and absolute alcohol have long been applied to treat intractable cancer pain and other incurable conditions.[1,16,17] The disadvantages of phenol and absolute alcohol are that these agents are highly destructive and possess weak or no local anesthetic activity. Alcohol produces an immediate progressive burning paraesthesia that gradually fades over several hours. Phenol elicits an immediate relief of the target pain that may gradually return in the ensuing few hours. The ultimate effect of the nerve lesion begins to appear 12-48 h after the injection of alcohol or phenol. We previously suggested that N-butyl tetracaine may be used as an alternative for phenol or alcohol application because of its strong local anesthetic-neurolytic dual properties.[4] Neurolytic tetracaine derivatives will elicit rapid local anesthesia when injected near the peripheral nerve and also cause nerve degeneration and therefore relieve the painful sensation for a prolonged period. It is possible that new neurolytic compounds with a higher diffusion rate through the cell membrane may have to be designed and synthesized before applications of these drugs for the other peripheral nerves, dorsal roots, or sympathetic ganglia become feasible. Besides general toxicity of these neurolytic compounds and the apparent lack of functional recovery in rats treated with N-hexyl tetracaine, other factors such as possible neuritis or neuroma formation after drug treatment also must be evaluated.

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