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Use of Bulleyaconitine A as an Adjuvant for Prolonged Cutaneous Analgesia in the Rat

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Abstract

BACKGROUND—Bulleyaconitine A (BLA) is an analgesic and antiinflammatory drug isolated from *Aconitum* plants. BLA has several potential targets, including voltage-gated Na⁺ channels. We tested whether BLA elicited long-lasting cutaneous analgesia, when co-injected with lidocaine and epinephrine, as a model for prolonged infiltration anesthesia.

METHODS—The local anesthetic properties of BLA were assessed by the patch-clamp technique in HEK293t cells expressing Nav1.7 and Nav1.8 neuronal Na⁺ channels, both crucial for nociception. Drug solutions (0.6 mL) were injected subcutaneously via rat shaved dorsal skin. Inhibition of the cutaneous trunci muscle reflex was evaluated by pinpricks. Skin cross-sections were stained with hematoxylin and eosin or with antibodies against PGP9.5.

RESULTS—BLA at 10 μM interacted minimally with resting or inactivated Nav1.7 and Nav1.8 Na⁺ channels when infrequently stimulated to +50 mV for 3 ms. However, when stimulated at 2 Hz for 1000 pulses, their peak Na⁺ currents were >90% reduced by BLA. This use-dependent inhibition was not significantly reversed after 15-min washing. Complete nociceptive blockade after injection of lidocaine (0.5%)/epinephrine (1:200,000) lasted for approximately 1 h in rats; full recovery occurred after approximately 6 h. Co-injection of 0.125 mM BLA with lidocaine/epinephrine increased the duration of complete nociceptive blockade to 24 h. Full recovery occurred after approximately 6 days. Skin histology including peripheral nerve fibers appeared unaffected by BLA.

CONCLUSIONS—BLA inhibits Nav1.7 and Nav1.8 Na⁺ currents in a use-dependent manner. Co-injection of BLA at ≤0.125 mM with lidocaine and epinephrine elicits complete cutaneous analgesia that lasts for up to 24 h without adverse effects.

Bulleyaconitine A (BLA) is isolated from *Aconitum* plants and classified as an “aconitine-like” alkaloid.¹ Aconitine not only inhibits neuronal Na⁺ currents during repetitive pulses but also shifts the voltage dependence of Na⁺ channel activation toward the hyperpolarizing direction and induces threshold Na⁺ currents near the resting potential.² BLA likewise reduces peak

neuronal Na⁺ currents by more than 90% after 1000 repetitive pulses.³ The shift in Na⁺ channel activation by BLA also occurs, but threshold Na⁺ currents appear minimal. Such a phenomenon could be due to a greater reduction of the single-channel conductance by BLA.⁴

After IV administration, most aconitine-like alkaloids cause cardiac arrhythmia and induce hyperexcitability.⁵ Despite these potential side effects, in China BLA in solution (0.2 mg/2 mL; IM) or in tablet (0.4 mg) has been prescribed for the treatment of chronic pain and rheumatoid arthritis. Among many possible *in vivo* targets, the central catecholaminergic and serotonergic systems are thought to be involved in analgesia induced by aconitine-like alkaloids.^{1,6,7} Recently, we reported that BLA when co-injected with lidocaine or epinephrine elicited complete sciatic nerve block that lasted for approximately 4 h in rats.³ However, it remains untested whether BLA, when injected subcutaneously, induces cutaneous analgesia or hyperexcitability.

In this study, we first examined the inhibition of Nav1.7 and Nav1.8 neuronal Na⁺ currents by BLA, since these channels are crucial for the excitability of peripheral nociceptive nerve fibers and are implicated as attractive targets for novel analgesic drugs.^{8–10} In addition, we determined the efficacy of BLA alone and as an adjuvant for cutaneous analgesia *in vivo*. We found that subcutaneous injection of BLA alone at ≥ 0.25 mM induced acute systemic side effects in rats, as detailed in the Results section. The systemic toxicity of BLA at ≤ 0.125 mM, however, was minimal. Unexpectedly, we found that cutaneous analgesia lasts for up to 6 days when BLA is co-injected with the lidocaine/epinephrine mixture subcutaneously. The implications and possible mechanisms of such prolonged cutaneous analgesia will be discussed.

METHODS

Drugs

BLA was purchased from Axxora, San Diego, CA. The BLA stock solution (50 mM) was prepared and stored at 4°C. Lidocaine-HCl 2.0% and epinephrine (1:1,000 or 1 mg/mL) vials were purchased from Abbott Laboratories, North Chicago, IL.

Transient Transfection

Human embryonic kidney cells (HEK293t) were grown in Ti-25 flasks to approximately 50% confluence in DMEM (Life Technologies, Rockville, MD) containing 10% fetal bovine serum (HyClone, Logan, UT), 1% penicillin and streptomycin solution (Sigma, St. Louis, MO), 3 mM taurine, and 25 mM HEPES (Life Technologies), and then transfected by calcium phosphate precipitation. Transfection of hNav1.7¹¹ and rNav1.8¹² Na⁺ channel clone (10 μ g) along with rat or human β 1-pcDNA1/Amp (20 μ g) and reporter CD8-pih3m (1 μ g) was adequate for current recording. We used hNav1.7 because it was available to us as a gift from Dr. Klugbauer. Cells were replated 15 h after transfection in 35-mm dishes, maintained at 37°C in a 5% CO₂ incubator, and used after 1–4 days. For cells expressing Nav1.8 channels, lidocaine (1 mM) was included in the medium as reported.¹³ Transfection-positive cells were identified with CD8-immunobeads (Dynabeads, Lake Success, NY).

Whole-Cell Voltage-Clamp Experiments

Whole-cell configuration was used to record Na⁺ currents of HEK293t cells at room temperature (21–24°C).¹⁴ Currents were acquired with an Axopatch 200B amplifier and pCLAMP 9 software (Molecular Devices, Sunnyvale, CA), filtered at 5 kHz, and sampled at 20 kHz. Borosilicate micropipettes were pulled (P-87, Sutter Instrument, Novato, CA) and heat-polished. Pipette electrodes contained 100 mM NaF, 30 mM NaCl, 10 mM EGTA, and 10 mM HEPES adjusted to pH 7.2 with CsOH. Access resistance was generally 1–3 M ω . Series

resistance was compensated by >85%; holding potential was set at -140 mV. The bath solution contained 65 mM NaCl, 85 mM choline Cl, 2 mM CaCl_2 , and 10 mM HEPES adjusted to pH 7.4 with tetramethylammonium hydroxide. Drugs were diluted with the bath solution and applied to the cell surface from a series of small-bore glass tubes.

Drug Injection and Neurobehavioral Examination

The protocol for animal studies was approved by the Harvard Medical Area Standing Committee on Animals. Male Sprague-Dawley rats (250–300 gm) were purchased from Charles River Laboratory (Wilmington, MA). All rats were briefly anesthetized by inhalation of 1%–2% sevoflurane (Abbott Laboratories; North Chicago, IL). Drug solutions freshly prepared in 0.9% NaCl saline were injected subcutaneously via the shaved dorsal thoracolumbar region. The injection resulted in a circular wheal, which was then marked with ink. Groups of six rats were injected with each test solution.

The cutaneous trunci muscle reflex (CTMR) was measured as a reaction to noxious pinprick.¹⁵ A Von Frey filament (26.0 g) was affixed to an 18-gauge needle and used to standardize the stimulus intensity. After observing the animal's normal reaction to six pinpricks applied on the contralateral control side, six pinpricks were then applied inside the wheal. No CTMR response after six pinpricks was defined as complete blockade (i.e., 100% of maximum possible effect [MPE]); three responses as 50% MPE; and six responses after six pinpricks as 0% MPE. The observer was blinded to the drugs used. Systemic side effects such as sedation, arrhythmia, respiratory distress, and hypothermia were also evaluated. For sedation: rats appeared immobile and restful when injected with 2% lidocaine. For arrhythmia: the rats' heart rates were monitored by an electrocardiogram monitor as needed. For respiratory distress: rats developed air hunger and discharged fluid from deep airways (as determined postmortem). For hypothermia: the body temperature was monitored by hand and compared with uninjected rats.

Skin Histology and Immunohistochemistry

For tissue toxicity, skin was excised from euthanized rats, fixed by 10% formalin, cross-sectioned, and stained with hematoxylin and eosin. To determine whether nerve degeneration occurred after the drug injection, we performed immunohistochemistry in the hindpaw and back skin using a PGP-9.5 antibody that stained all nerve fibers.¹⁶ Rats were killed with isoflurane and perfused through the ascending aorta with saline followed by 4% paraformaldehyde. Skins were dissected, postfixed in the same fixative overnight, and cross-sectioned in a cryostat (30 μm in thickness). All sections were first blocked with 2% control serum for 1 h at room temperature and incubated overnight at 4°C with primary antibody against PGP9.5 (antirabbit, 1:1000, Biogenesis, Sandown, NH). The sections were then incubated for 1 h at room temperature with Cy3-conjugated secondary antibody (1:400). Finally, the sections were mounted on slides, coverslipped with a medium containing p-phenylenediamineglycerol, and examined by a fluorescence-microscope equipped with a CCD digital camera. The times of animal termination in these experiments are specified in the figure legends and all sections were coded in a blinded fashion.

Statistical Analysis

An unpaired Student's *t*-test was used to evaluate the degree of Na^+ channel block. *P* values of <0.05 were considered statistically significant. One-way analysis of variance was used to evaluate the significance of differences in the CTMR block, and in the area under the curve (AUC) among groups. Given a significant *F* test ($P < 0.05$), a Bonferroni adjustment was performed to obtain *post hoc* pairwise tests for contrasts of interest. Because of the ordinal categorical nature of the block scores, an overall test for drug effect was also obtained via generalized estimating equations.¹⁷ A cumulative logistic ordinal model was fit with a linear

and quadratic trend in time and time by group interaction. The overall *P* value was calculated via PROC GENMOD (SAS 9.1, Carey, NC).

RESULTS

BLA at 10 μ M Did Not Interact with Resting or Inactivated States of Nav1.7 and Nav1.8 Na⁺ Channels

Figure 1A shows traces of outward Na⁺ currents recorded at +50 mV from Hek293t cells expressing Nav1.7 Na⁺ channels before (solid trace) and after (dotted trace) external application of 10 μ M BLA for 5 min. The Na⁺ currents were outward because the intracellular solution contained 130 mM Na⁺ ions.¹⁸ Recordings of outward Na⁺ currents minimized the access resistance artifact due to positive feedback cycle. The direction of Na⁺ currents is governed by the Na⁺ reversal potential and has little influence on Na⁺ channel function. No effects of BLA on either the peak amplitude or current-decaying kinetics were evident when the cell was stimulated once every 30 s. The ratio of peak currents before and after 10 μ M BLA was $97.3 \pm 1.5\%$ ($n = 6$), demonstrating that BLA did not block the resting Na⁺ channels at the -140 mV holding potential.

Since local anesthetics interact preferentially with inactivated Na⁺ channels,¹⁹ we tested whether BLA also displayed such a preference for inactivated Nav1.7 Na⁺ channels. We applied a conditioning pulse of -70 mV for 10 s to allow fast-inactivated Na⁺ channels to interact with 10 μ M BLA (Fig. 1B), as shown for local anesthetics with this pulse protocol.^{20,21} Under this condition, BLA did not inhibit the peak hNav1.7 Na⁺ currents, nor did it affect the fast-decaying kinetics of Nav1.7 Na⁺ currents. The ratio of peak currents before and after 10 μ M BLA was $99.5 \pm 1.9\%$ ($n = 5$) for inactivated Nav1.7 channels. We therefore conclude, that BLA does not interact with resting or inactivated Nav1.7 Na⁺ channels significantly. Similar results were observed for the resting and inactivated Nav1.8 Na⁺ channels as shown in the bottom panels of Figure 1 C and D, respectively. A conditioning pulse of -40 mV for 10 s (Fig. 1D) was used for the inactivated rNav1.8 Na⁺ channels as reported earlier.¹² The ratio of peak currents before and after 10 μ M BLA was $96.4 \pm 2.2\%$ ($n = 5$) for resting Nav1.8 Na⁺ channels and $96.7 \pm 3.7\%$ ($n = 5$) for inactivated Nav1.8 Na⁺ channels. Preliminary results showed that an increase of BLA concentration to 100 μ M did not produce much block (<10%) of inactivated Na⁺ channels. This small block could be due to the block of the open Na⁺ channels during the test pulse as described next.

BLA Inhibited Nav1.7 and Nav1.8 Na⁺ Currents in a Use-Dependent Manner

When the cell was stimulated at 2 Hz, however, Nav1.7 Na⁺ currents were progressively reduced by 10 μ M BLA. Figure 2A shows selected traces of Nav1.7 Na⁺ currents at +50 mV with 1000 repetitive pulses. Peak Na⁺ currents decreased progressively until they reached a steady-state level near 1000P. The amount of reduction by 10 μ M BLA was $91.1 \pm 2.0\%$ ($n = 5$) after 1000 repetitive pulses. This use-dependent block was not significantly reversed after 15-min washing with the drug-free solution; $89.7 \pm 1.0\%$ ($n = 5$) of Na⁺ currents remained unaccountable. In contrast, use-dependent block by lidocaine or bupivacaine reversed fully within 1 min even without washout.²²

Under identical conditions, BLA at 10 μ M inhibited peak Nav1.8 Na⁺ currents by $91.5 \pm 1.2\%$ ($n = 7$) (Fig. 2B) after 1000 repetitive pulses. Most of the late Na⁺ currents were proportionally reduced during repetitive pulses. Once it occurred, this use-dependent inhibition of Nav1.8 Na⁺ currents was also not significantly reversed after 15-min washing with the drug-free solution; $89.0 \pm 2.4\%$ ($n = 7$) of Na⁺ currents remained unaccountable. Control experiments in the absence of BLA showed little reduction of Nav1.7 and Nav1.8 peak currents during repetitive pulses. These results suggested that BLA interacted only with open states of Nav1.7

and Nav1.8 Na⁺ channels in a use-dependent manner. Such a binding phenomenon was commonly found among Na⁺ channel activators.^{4,23}

Cutaneous Analgesia and Side Effects Induced by Single Injections of BLA

After subcutaneous injections of 0.125 mM BLA in a volume of 0.6 mL via the dorsal surface of the thoracolumbar region, complete nociceptive blockade in the CTMR assay lasted for approximately 3 h in rats (Fig. 3; open squares). Onset for cutaneous analgesia reached complete blockage approximately 30 min after injection; full recovery occurred in approximately 24 h. No obvious systemic side effects were evident during the nociceptive blockade period except that 2 of 6 rats had mild diarrhea approximately 1 h after injection. When injected with 0.25 mM BLA (0.6 mL), rats appeared sedated, and developed various systemic side effects as judged by clinic observations: arrhythmia, respiratory distress, and hypothermia approximately 1.5 h after injection. Four of 6 rats died approximately 2 h after injection. When injected with the concentration of 0.5 mM BLA, all rats (3/3) died approximately 1–2 h after injection.

Coadministration of BLA with Lidocaine and Epinephrine

To prolong cutaneous analgesia, we tested a solution containing a mixture of 0.125 mM BLA/0.5% lidocaine/1:200,000 epinephrine. Epinephrine is a vasoconstrictor that limits blood flow in tissue, but its effects usually last only for a couple of hours. Unexpectedly, co-injection of 0.125 mM BLA with lidocaine (0.5%)/epinephrine (1:200,000) increased the duration of complete nociceptive blockade from 3 to 24 h; full recovery occurred after 6 days (Fig. 3; closed versus open square). Furthermore, we found no apparent systemic side effects of BLA in treated rats. In a control experiment, lidocaine (0.5%) plus epinephrine (1:200,000) produced a short complete nociceptive blockade, approximately 1 h in duration (Fig. 3; open circles). Epinephrine itself at 1:200,000 induced partial, transient analgesia after subcutaneous injections, but its effects were cleared within 2 h.²⁴

Figure 4 shows the dose-response curves of BLA plus the 0.5% lidocaine/1:200,000 epinephrine mixture. These dose-response data indicated that the higher the BLA concentration (ranging from 0.05 to 0.125 mM) in this drug mixture, the longer the duration of the complete cutaneous analgesia. Figure 5 shows the time for full recovery from cutaneous analgesia under various drug combinations. Interestingly, the duration to full recovery remained relatively constant with BLA concentrations at 0.075, 0.1, and 0.125 mM (lasting for 144 h or 6 days). To compare the overall drug-combination effects in cutaneous analgesia, we measured their areas under the curve (AUC; %MPE * time). Table 1 shows that the AUCs for the BLA/lidocaine/epinephrine solution was 1403.7 when 0.05 mM BLA was tested, whereas the AUCs were measured 6211.0, 7019.5, 8125.5, respectively, when 0.075, 0.1, and 0.125 mM were included. AUCs for 0.125 mM BLA/0.5% lidocaine and for 0.075 mM BLA/1:200,000 epinephrine were measured 1035.5 and 5015.5, respectively (Table 1). These results suggested that epinephrine was more important than lidocaine for the long-lasting cutaneous analgesia induced by BLA/lidocaine/epinephrine.

Coadministration of BLA with Lidocaine

To test whether lidocaine minimizes the systemic side effects of BLA, we co-administered 0.125 mM or 0.25 mM BLA along with 0.5% lidocaine subcutaneously. Onset for cutaneous analgesia was rapid and reached its maximal effects within 5–10 min, probably due to the rapid action of 0.5% lidocaine. Complete nociceptive blockade lasted for 3–6 h; full recovery occurred in 24 h. This duration to full recovery from nociceptive blockade was not longer than that induced by 0.125 mM BLA alone (Fig. 5 and Table 1). Systemic adverse effects with arrhythmia, sedation, and hypothermia occurred approximately 1.5 h after injection of 0.25 mM BLA/0.5% lidocaine, but these adverse effects were less severe than those induced by the

injection of 0.25 mM BLA alone. Only one of six rats died approximately 9 h after injection, from respiratory failure (versus 4 of 6 rats died approximately 2 h after injection with 0.25 mM BLA alone). Thus, coinjection of BLA with lidocaine lessened the severity of the systemic side effects induced by 0.25 mM BLA but did not appear to prolong the time to full recovery from cutaneous analgesia.

Lack of Tissue Toxicity by BLA

Tissue toxicity after co-injection of BLA 0.1 to 0.125 mM along with 0.5% lidocaine and 1:200,000 epinephrine was examined in cross-sections of the rats' skin tissue by a pathologist (Dr. Berigitta Schmidt). There were no apparent changes in histology compared with the control sections, except for mild lymphocytic perivascular infiltration approximately 6 days after co-injection (Fig. 6). Inflammation scores in and around the injected area, however, were not significantly different from the control. Furthermore, we observed no apparent loss in PGP9.5 immunostained peripheral nerve fibers in both the back and paw skins approximately 1 day after co-injection (Fig. 7). The number of nerve fibers in each optical field for paw skins, for example, ranged 18–24 (mean $\pm$ SE = 21.3 ± 0.7 , in 9 sections from 3 animals) in the saline group and 19–23 (21.6 ± 0.5 , $P = 0.732$) in the BLA-treated group.

DISCUSSION

We have demonstrated that BLA is a potent use-dependent blocker for both Nav1.7 and Nav1.8 Na⁺ currents and that subcutaneous injection of BLA as an adjuvant to lidocaine/epinephrine drastically prolongs the duration of cutaneous analgesia. The significance and implications of these findings are discussed as follows.

BLA as a Potent Use-Dependent Blocker for Nav1.7 and Nav1.8 Na⁺ Channel Isoforms

Nav1.7 and Nav1.8 Na⁺ channels are expressed predominantly in nociceptive neurons within dorsal root ganglia.⁸ Nav1.7 is likely responsible for a threshold current near the resting potential,²⁵ whereas Nav1.8 is responsible for most of the current underlying the action potentials in nociceptive neurons.²⁶ Accumulated evidence supports the notion that these two isoforms are essential for the development of acute, inflammatory, and neuropathic pain.^{9, 10} BLA at 10 μ M potently inhibits both Nav1.7 and Nav1.8 Na⁺ currents during repetitive pulses but interacts minimally with resting or inactivated Nav1.7 and Nav1.8 Na⁺ channels. Previously, we have also demonstrated that BLA drastically reduced neuronal Na⁺ currents during repetitive pulses in GH₃ cells, which expressed neuronal Nav1.1, 1.2, 1.3, and 1.6 Na⁺ channels.²⁷ In fact, all the blocking phenotypes induced by BLA in Nav1.7 and Nav1.8 isoforms are similar to those found in GH₃ cells. We therefore conclude that BLA is nonselective for these neuronal Na⁺ channels, including isoforms responsible for nociception.

Unlike other Na⁺ channel activators (e.g., batrachotoxin and veratridine),^{4,23} BLA does not simultaneously elicit persistent late Na⁺ currents during repetitive pulses whereas the peak Na⁺ currents are reduced (Fig. 2). This phenotype implies that 1) BLA preferentially binds with the open state of Nav1.7 and Nav1.8 Na⁺ channels, 2) fast Na⁺ channel inactivation still reaches its completion, and 3) upon binding, BLA reduces their single-channel conductance but more so than batrachotoxin and veratridine. The single-channel conductance in the presence of BLA may be miniscule, since BLA inhibits >90% peak Na⁺ currents after 1000 repetitive pulses. There is little reversal of the use-dependent inhibition of Nav1.7 and Nav1.8 Na⁺ currents after 15-min washing. This slow-reversal phenomenon suggests that BLA dissociates rather slowly from its binding site.

Use of BLA as an Adjuvant in the Lidocaine/Epinephrine Mixture for Prolonged Cutaneous Analgesia

With a single injection, BLA (0.6 mL at 0.125 mM) elicits complete cutaneous block that lasts for 3 h. However, when BLA is co-injected with lidocaine (0.5%)/epinephrine (1:200,000) the complete cutaneous block lasts for up to 24 h (Fig. 3). It seems puzzling that BLA prolongs cutaneous analgesia so drastically when co-injected with lidocaine/epinephrine. The action of lidocaine/epinephrine cannot account for such prolongation, since their effects wear off within 6 h (Fig. 3). In particular, lidocaine alone did not prolong cutaneous analgesia induced by BLA (Fig. 5). Four alternative explanations could be considered for this synergistic action. First, BLA may be “trapped” within the skin tissue which then acts as a reservoir for its slow release. Such BLA trapping may be greatly enhanced by epinephrine. This mechanism is equivalent to the slow release of haloperidol decanoate after IM injection²⁸ and implies that BLA remains near the site of injection for as long as 24 h, so that cutaneous analgesia persists. Second, BLA may bind tightly with its receptor within Na⁺ channels and does not dissociate from the receptor site readily. In our view, the tight BLA binding with Na⁺ channels likely operates as a supporting role in prolonged anesthesia/analgesia. This is because the complete sensory block by 0.125 mM BLA alone lasts no longer than 3 h (Fig. 3; open square), whereas the complete sensory block by the BLA/lidocaine/epinephrine mixture lasts for 24 h (Fig. 3; closed square). Third, trapped BLA may affect the level of Na⁺ channels within the plasma membrane (e.g., channel expression or degradation). Finally, the fourth possible explanation is that trapped BLA damages the skin tissue, including nociceptive nerve fibers; however, as discussed next, we observed no apparent tissue toxicity after BLA injection.

Potential Tissue Toxicity and Side Effects After Cutaneous Injection of BLA as an Adjuvant

Even though cutaneous analgesia lasted for several days after co-injection of BLA/lidocaine/epinephrine, we observed no tissue damage in the cross-sections of the skin surrounding the injected site. The epidermis, dermis, and blood vessels all appeared intact (Fig. 6). Mild lymphocytic perivascular infiltration was noted but inflammation scores were not significantly different from the control. Furthermore, the possibility that BLA exhibited selective toxicity towards peripheral nerve fibers was excluded by PGP 9.5 immunostaining. The number of peripheral nerve fibers remained unchanged one day after BLA injection (Fig. 7).

One documented drawback of using BLA and other aconitine-like alkaloids is their narrow therapeutic index.⁵ However, this shortcoming may become less serious at lower BLA concentrations. The systemic side effects were minimal in rats when the concentration of BLA injected was ≤ 0.125 mM; all rats behaved normally during the entire observation period. In addition, we found that subcutaneous co-injection of lidocaine and epinephrine also reduced the systemic side effects of BLA, probably due to vasoconstriction.

Possible BLA Applications in Infiltration Anesthesia

BLA at ≤ 0.125 mM is effective for producing cutaneous analgesia with minimal adverse effects. Lidocaine may be added for rapid on-set in cutaneous analgesia, whereas epinephrine may be added to lengthen its duration and/or to reduce BLA dosage (Fig. 5). Prolonged cutaneous analgesia induced by BLA as an adjuvant could have important clinical applications. First, long-lasting cutaneous analgesia may be beneficial for patients with postoperative pain.^{29,30} Second, BLA possesses anti-inflammatory properties that may have added benefits for patients with inflammation after surgery. Third, prolonged cutaneous analgesia may also alleviate neuropathic pain, which is likely caused by high-frequency discharges.^{31–33} Additional studies using various pain models will be needed to test these possibilities.

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Figure 1.

Resting and inactivated Nav1.7 and Nav1.8 channels are insensitive to 10 μM bulleyaconitine A (BLA). Superimposed traces of Nav1.7 and Nav1.8 Na^+ currents were recorded before (solid trace) and 5 min after application of 10 μM BLA (dashed trace). For the resting Nav1.7 (A) and Nav1.8 (C) block by BLA, the cell was held at -140 mV and stimulated once every 30 s by a brief test pulse ($+50$ mV for 3 ms). For the inactivated Nav1.7 (B) and Nav1.8 (D) block by BLA, the cell was depolarized to -70 mV and -40 for 10 s, respectively. An interpulse (-140 mV for 100 ms), which allowed drug-free inactivated Na^+ channels to recover, was applied before the brief test pulse.

A Use-dependent block of Nav1.7 by BLA **B** Use-dependent block of Nav1.8 by BLA **Figure 2.**

Use-dependent block of Nav1.7 and Nav1.8 Na⁺ currents by Bulleyaconitine A (BLA). (A) Superimposed traces of Nav1.7 Na⁺ currents were recorded in the presence of 10 μ M BLA with 1000 repetitive pulses (+50 mV for 4 ms) applied at 2 Hz. The cell was perfused with 10 μ M BLA approximately 5 min before repetitive pulses. Current traces are labeled with the number of corresponding pulses, ranging from 1P to 1000P. (B) Superimposed traces of Nav1.8 Na⁺ currents were recorded in the presence of 10 μ M BLA with 1000 repetitive pulses. The conditions were the same as described in (A). Control experiments in the absence of BLA showed little reduction of Nav1.7 or Nav1.8 peak currents during repetitive pulses.

Figure 3.

Cutaneous analgesia induced by Bulleyaconitine A (BLA) alone or as an adjuvant. The cutaneous trunci muscle reflex (CTMR) was determined as described in Methods. In each group, six rats were tested and their % maximum possible effect (MPE) $\pm$ SE was plotted against the corresponding time interval. Subcutaneous injection of 600 μ L lidocaine (0.5%)/epinephrine (1:200,000) produced a complete CTMR block (100% MPE) lasting for approximately 1 h (open circle). The CTMR block recovered fully after 6 h. With 600 μ L of BLA at 0.125 mM, the complete CTMR block lasted for approximately 3 h and recovered fully after approximately 24 h (open square). With co-injection of 600 μ L of BLA 0.125 mM/lidocaine (0.5%)/epinephrine (1:200,000), the complete CTMR block lasted for approximately 24 h and recovered fully after approximately 144 h or approximately 6 days (closed square).

Figure 4.

Dose-response curve of Bulleyaconitine A (BLA) as an adjuvant in cutaneous analgesia. BLA at various concentrations (0.05–0.125 mM) was co-injected with lidocaine (0.5%) and epinephrine (1:200,000). Cutaneous trunci muscle reflex (CTMR) was measured at various intervals as described in the Methods section. At each BLA concentration, six rats were tested and their % maximum possible effect (MPE) $\pm$ SE was plotted against the corresponding time interval.

Figure 5.

Time to full recovery from cutaneous analgesia after injections of various solutions. In each group, six rats were injected and their full recovery times ($h \pm se$) from cutaneous analgesia were tested, as described in Methods, and plotted against the drug injected. One-way analysis of variance was used to test for overall mean differences in time to full recovery from cutaneous analgesia among 1) Bulleyaconitine A (BLA) 0.125 mM, BLA 0.075 mM+Epi (epinephrine; 1:200,000), and BLA 0.075 mM+Lido/Epi (0.5% lidocaine and 1:200,000 epinephrine); 2) BLA 0.125 mM, BLA 0.125 mM+lidocaine 0.5%, and BLA 0.125 mM+Lido/Epi; and 3) mixtures of Lido/Epi with BLA ranging from 0.050 to 0.125 mM. Given a significant F test ($P < 0.05$), pairwise *post hoc* Bonferroni tests were performed for multiple pairwise comparisons. 1) F value was 27.5 with $P < 0.001$. But there was no significant difference between BLA 0.075 mM+Epi and BLA 0.075 mM+Lido/Epi. 2) F value was 334.1 with $P < 0.001$. $**P < 0.001$, compared with the BLA 0.125 mM group. There was no significant difference between BLA 0.125 mM and BLA 0.125 mM+lidocaine 0.5%. 3) F value was 48.4 with $P < 0.001$. $++P < 0.001$, compared with the BLA 0.05 mM+Lido/Epi group.

Figure 6.

A representative light micrograph of the skin tissue after Bulleyaconitine A injection. Rat shaved dorsal skin was injected with 0.6 mL of 0.1 mM Bulleyaconitine A/0.5% lidocaine/1:200,000 epinephrine. After full recovery from cutaneous analgesia (approximately 6 days), the skin tissue around the injected site was processed and hematoxylin and eosin stained as described in the Methods. The epidermis layer of the skin is shown on top, followed by the dermis, muscle, and subcutaneous layer. Magnification: 4 $\times$; inset, 40 $\times$. The circles in the inset indicate examples of the mild lymphocytic perivascular infiltration within the subcutaneous layer.

Figure 7. PGP9.5 immunostaining of rat skin. (A) Paw skin, low magnification; saline (left panel), control side; Bulleyaconitine A (BLA) (right panel), injected side (0.1 mL of 0.125 mM BLA/0.5% lidocaine/1:200,000 epinephrine for paw or 0.6 mL for back skins). Skin samples were taken approximately 24 h after injection. (B) Paw skin, high magnification; (C) Back skin, high magnification. Nonspecific staining (*) on the skin surface was also present. Arrows indicate nerve fibers in the epidermis of the skin. Scale bars, 50 μ m.

Table 1

Relative Values of the Area Under the Curve (AUC; MPE% * h) Versus Drug Combinations

Drug combinations	AUC (mean $\pm$ SEM, $n = 6$)
0.5% Lidocaine/Epinephrine	394.8 $\pm$ 17.1 ^{*†}
0.125 mM BLA	940.0 $\pm$ 99.0
0.125 mM BLA/0.5% Lidocaine	1035.5 $\pm$ 108.4
0.075 mM BLA/Epinephrine	5015.5 $\pm$ 1113.2 [†]
0.050 mM BLA/Lidocaine/Epinephrine	1403.7 $\pm$ 221.8
0.075 mM BLA/Lidocaine/Epinephrine	6211.0 $\pm$ 568.7 ^{†‡}
0.100 mM BLA/Lidocaine/Epinephrine	7019.5 $\pm$ 630.8 ^{†‡}
0.125 mM BLA/Lidocaine/Epinephrine	8125.5 $\pm$ 464.6 ^{†‡}

Most data are derived from Figure 3 and Figure 4 and presented as mean $\pm$ SEM. The control solution (0.5% lidocaine/1:200,000 epinephrine) has a significantly smaller AUC than all other test solutions (

^{*} $P < 0.05$). An unpaired Student's t -test was used to test for a difference between each drug combination and 0.125 mM bulleyaconitine A (BLA).

[†] $P < 0.05$. No significant difference was found among 0.125 mM BLA, 0.125 mM BLA/0.5% lidocaine, and 0.05 mM BLA/lidocaine/epinephrine. One-way analysis of variance was used to test for overall mean differences in the area under the curve among mixtures of lidocaine/epinephrine, with BLA ranging from 0.050 to 0.125 mM. Given a significant F test (F value = 35.6) with $P < 0.001$, pairwise *post hoc* Bonferroni tests were performed for multiple pairwise comparisons.

[‡] $P < 0.001$, compared with the 0.050 mM BLA/lidocaine/epinephrine group. The GEE statistic analyses likewise supported these conclusions.