Vanilloid Receptor Agonists Potentiate the In Vivo Local Anesthetic Activity of Percutaneously Injected Site 1 Sodium Channel Blockers

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Background: Capsaicin, the pungent ingredient in chili peppers, is a vanilloid with noxious and analgesic effects that inhibits tetrodotoxin-resistant sodium currents. Because tetrodotoxin-resistant currents are found primarily in small-diameter nociceptor afferents of the peripheral nerves, their inhibition may lead to selective analgesia. Therefore, the authors evaluated the interactions between tetrodotoxin, a site 1 sodium channel blocker, and capsaicin on nociceptive neurons in vivo.

Methods: Percutaneous sciatic nerve injections with 0.9 to 9.9 nm capsaicin, 0.0 to 120 μg tetrodotoxin, or both were administered to male Sprague-Dawley rats. Thermal nociceptive and motor blockades were measured. Data were expressed as medians with 25th and 75th percentiles.

Results: Capsaicin produced a transient increase in thermal latency with no effect on motor strength. Tetrodotoxin reduced motor strength for a longer duration than nociception. The interaction between tetrodotoxin and capsaicin was synergistic, as evidenced by (1) supradiadicutive prolongation of both nociceptive and motor block, with the effect of capsaicin reversed by the vanilloid antagonist capsazepine, and (2) synergism in the frequency that rats achieved maximal block shown by isobolographic analysis. The combination of tetrodotoxin and capsaicin showed less motor predominance than tetrodotoxin did alone. Similar interactions were found between tetrodotoxin and resiniferatoxin (another vanilloid), and between capsaicin and saxitoxin (another site 1 sodium channel blocker), but much less so between bupivacaine and capsaicin.

Conclusions: Site 1 sodium channel blockers and vanilloids have synergistic effects on nerve blockade in vivo. These interactions may be useful in developing prolonged local anesthetics and elucidating mechanisms of functionally selective nerve blockade. (Key words: Isobologram; median effective concentration [EC₅₀]; pain.)

CAPSAICIN (8-methyl-N-vanillyl-6-nonemide), the pungent ingredient in chili peppers, has been the focus of considerable interest because of its therapeutic potential as an analgesic agent and as a pharmacologic tool in research of excitation, desensitization, and neurotoxicity. Its effects on peripheral nerves also have been studied extensively. Jancsó et al. applied 1% capsaicin to the rat sciatic nerve and found an impairment of thermal nociception that lasted for days but that did not impair motor function. Fitzgerald and Woolf also documented long-lasting changes in thermal nociception after applying 1.5% capsaicin in 10% ethanol to the rat sciatic nerve, with no effect on motor function. The electrophysiologic effects of capsaicin also have been evaluated. Godfraind et al. showed that 30 mm capsaicin prolonged the action potential of the cultured chick sensory neuron, and that this effect was not affected by micromolar tetrodotoxin. Gamse et al. reported a marked diminution in compound action potentials when 1% capsaicin in an emulsion of 10% Tween 80 in paraffin oil was applied topically to the rat sciatic nerve. They did not observe recovery during 2 h of observation. Yamanaka et al. applied varying concentrations of capsaicin in 0.3% ethanol to isolated crayfish giant axon and found that the magnitude and rate of increase of the...
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action potential was suppressed. They found conduction block to be reversible and primarily attributable to the inhibition of sodium channels.

Tetrodotoxin, saxitoxin, and similar compounds block sodium channels at a site on the external surface of the cell that is called site 1 according to the classification of Catterall. Recently, investigators showed that capsaicin can affect dorsal root ganglion neurons that express tetrodotoxin-resistant sodium channels, and that some C fibers are capsaicin insensitive. Recently, Großkreutz et al. showed that 1 μM capsaicin blocked the tetrodotoxin-resistant sodium spike in isolated human sural nerve and reduced the C fiber component of the compound action potentials by 30 to 60%. The remaining component was not reduced further by higher levels of capsaicin, but it was blocked by 1 μM tetrodotoxin.

These findings prompted us to try to determine whether tetrodotoxin and capsaicin, which appear to affect pharmacologically distinct types of sodium currents, have any interaction when injected together to produce sciatic nerve blockade. Given the concerns regarding the C fiber-specific neurotoxicity of capsaicin, we performed most of our experiments at much lower concentrations of capsaicin (33 to 990 μM, or 0.001 to 0.03%) than were used in previous studies (in those, 1 to 1.5% capsaicin typically were used). We recently evaluated the clinically useful dosage range for tetrodotoxin in the rat. Tetrodotoxin has been shown to have no local neurotoxic effects.

Materials and Methods

Stock Solutions

Tetrodotoxin (Sigma Chemical Co., St. Louis, MO) and saxitoxin (Sigma Chemical Co.) were dissolved in 20 mM citrate buffer (sodium citrate/citrate, 55:45), pH 4.45, to a concentration of 0.1 μg/μl (0.3 mM). Solutions were then diluted in 154 mM NaCl as needed. Capsaicin (Sigma Chemical Co.) was dissolved in the detergent Tween 80 (Sigma Chemical Co.) at 55°C to a concentration of 100 μg/μl (330 mM). These solutions were stored at 4°C. Another vanilloid, resiniferatoxin (Sigma Chemical Co.), was dissolved in dimethyl sulfoxide (Sigma Chemical Co.) to a concentration of 1 μg/μl (1.6 mM) and stored at −80°C under nitrogen. Dilutions of solutions containing capsaicin or resiniferatoxin in 0.9% saline were maintained in 0.3% (vol/vol) Tween 80 to avoid precipitation (in the case of resiniferatoxin) and to facilitate comparison between experiments. Solutions containing more than 990 μM capsaicin contained higher percentages of Tween 80 because of the relatively small degree to which the original stocks could be diluted. Capsazepine was dissolved in dimethyl sulfoxide (5 mg/ml, 13.3 mM) and diluted as necessary. Bupivacaine hydrochloride (Astra USA, Westborough, MA) was obtained in 0.5% solution and diluted to the desired concentration.

Animal Care

Young adult male Sprague-Dawley rats weighing 310–420 g were used. Animals were cared for in compliance with protocols approved by the Animal Care and Use Committee at Children’s Hospital. Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). They were housed in groups and kept in a 6 a.m. to 6 p.m. light-dark cycle. Rats were not given repeated injections with capsaicin- or resiniferatoxin-containing solutions.

Sciatic Blockade Technique

Before rats received nerve block injections, they were anesthetized briefly with halothane by face mask. A 23-gauge needle was introduced posteromedially to the greater trochanter, pointing in an anteromedial direction. After bone was contacted, the needle was withdrawn 1 mm and 0.1 ml of drug-containing solution was injected. The left leg was always used for blocks, and the right leg served as the control.

Assessment of Nerve Blockade

The effectiveness of block was measured at various times, applying the methods of Thalhammer et al. or a modification of their methods.

Nociceptive block was assessed by a modified hot plate test. Hind paws were exposed in sequence (left then right) to a hot plate at 56°C (model 39D Hot Plate Analgesia Meter; IITC Inc., Woodland Hills, CA), and the time (latency) until paw withdrawal was measured by a stopwatch. If the paw remained in contact for 12 s, it was removed by the experimenter to avoid injury to the animal or the development of hyperalgesia. This test was repeated three times for each rat at every time point.

Motor strength was assessed by holding the rat with its posterior placed above a digital balance and allowing it to bear weight on one hind paw at a time. The maximum weight that the rat could bear without its ankle touching the balance was recorded. This measure is called the extensor postural thrust.
Data Processing

The data for nociceptive block were reported in terms of thermal latency and duration of block. The duration of thermal nociceptive block was the time required for thermal latency to return to a value of 7 s (which is 50% of maximal block when a baseline thermal latency of approximately 2 s was taken into account). The duration of motor block was defined as the time for weight bearing to return halfway to normal from maximal block.

The few animals that did not survive (rarely, 120 μM tetrodotoxin was fatal) were not included in the calculation of block duration, nor were those whose thermal latency did not return to less 7 s within 24 h (i.e., those in whom initial nerve block could not be distinguished from the secondary increase in latency, called phase II). All other animals were included in the calculations of median durations of block, including those in which blockade was unsuccessful, defined as injections that did not result in a thermal latency of at least 7 s or an extensor postural thrust suppression of at least 50%. The duration of block for the appropriate function was considered 0 (zero) for those injections. These zero-duration blocks probably resulted from real pharmacologic differences between drugs rather than operator error, because our rate of successful blockade has been more than 99% when we have used 0.5% bupivacaine with the same volume and method of blockade. Success was defined in the same manner for phase II (i.e., achievement of a thermal latency of at least 7 s).

Because some experimental groups had many zero-duration blocks (i.e., the frequency of block durations was not normally distributed), we used the median as the measure of central tendency throughout this report, with 25th and 75th percentiles (noted in parentheses). A median or 75th percentile value of zero does not necessarily mean that all blocks were unsuccessful.

Statistical Analysis

Statistical inferences on medians were made using the Kruskal–Wallis test, the Mann–Whitney U test (paired in comparisons between functional modalities in the same rat, unpaired in all other cases), or both. When multiple comparisons were made, Mann–Whitney U tests were only performed if intergroup differences were first detected using a Kruskall–Wallis test with P < 0.05. For the sake of readability, however, only the results of Mann–Whitney U tests are reported. A P value < 0.05 indicated significance, and the Bonferroni correction was used when many comparisons were made. Logit (logistic regression) analyses were used to derive median effective concentration (EC₅₀) values. These data analyses were conducted using Stata statistical software (Stata Corporation, College Station, TX).

Results

Local Anesthetic Properties of Capsaicin Alone

Deficits in thermal nociception after injection of capsaicin were characterized by a biphasic response (fig. 1). At low concentrations (<1.65 μM), injection resulted in either no block or a rapid-onset (within 10 min), transient elevation in thermal latency with no effect on the other functions tested. This initial event is called a phase I block. The duration of phase I varied with the concentration of capsaicin (table 1). Phase I was followed, in some rats, by a secondary increase in thermal latency (phase II), without any change in motor performance (fig. 1). The delay between phase I and phase II was in approximate inverse relation to the concentration used: there were no phase II blocks in 12 rats injected with 350 μM capsaicin, whereas there were 12 in the 15 rats injected with 9.9 μM capsaicin. At high doses the two were often merged (this was uniformly the case at 9.9 μM capsaicin). The duration of this prolonged thermal-selective analgesia varied (from 1 to 9 days) and was in
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Table 1. Duration of the Phase I for Thermal Nociception from Capsaicin, with or without 90 μM TTX

<table>
<thead>
<tr>
<th>Capsaicin Alone</th>
<th>Capsaicin (μM)</th>
<th>Duration (min)</th>
<th>n</th>
<th>Capsaicin + 90 μM TTX</th>
<th>Capsaicin (μM)</th>
<th>Duration (min)</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 (0–0)</td>
<td>12</td>
<td></td>
<td>0</td>
<td>88 (24–118)</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>0 (0–0)</td>
<td>6</td>
<td></td>
<td>33</td>
<td>179 (129–253)</td>
<td>11</td>
<td></td>
<td>.03</td>
</tr>
<tr>
<td>99</td>
<td>0 (0–0)</td>
<td>6</td>
<td></td>
<td>99</td>
<td>270 (115–344)</td>
<td>12</td>
<td></td>
<td>0.0025</td>
</tr>
<tr>
<td>330</td>
<td>0 (0–0)</td>
<td>12</td>
<td></td>
<td>330</td>
<td>201 (120–248)</td>
<td>12</td>
<td></td>
<td>0.0097</td>
</tr>
<tr>
<td>990</td>
<td>0 (0–0)</td>
<td>24</td>
<td></td>
<td>990</td>
<td>289 (169–329)</td>
<td>12</td>
<td></td>
<td>0.0007</td>
</tr>
<tr>
<td>1650</td>
<td>0 (0–14)</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2475</td>
<td>49 (0–69)</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3300</td>
<td>52 (11–77)</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4950</td>
<td>62 (0–77)</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>66000</td>
<td>98 (63–117)</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Durations are medians with 25th and 75th percentiles. P values are the result of Mann-Whitney U tests comparing combinations of capsacin + 90 μM TTX with 90 μM TTX alone. Since there are five comparisons, P < 0.01 is considered significant.

approximate proportion to the dose of capsaicin administered. In some rats, thermal analgesia did not return to baseline and lasted for more than 8 months (the longest period that we observed such rats). Phase II is not the focus of this study, and has been described elsewhere,7 so our remarks on this phenomenon will be limited. At 9.9 mM (the highest dose), all functions of the injected leg were impaired transiently. However, the contralateral limb was affected to a similar degree, suggesting that this was a result of systemically distributed capsacin rather than a specific nerve block. Bilateral suppression of motor strength was also seen in rats given this dose of capsacin subcutaneously at a site remote from the sciatic nerve (data not shown).

As noted before, capsacin has neurotoxic effects when used at high concentrations. Because phase II may have corresponded to actual nerve damage, we conducted most of the experiments by combining tetrodotoxin and capsacin at capsacin concentrations that did not commonly cause phase II; that is, at 1 mM and less (990 μM capsacin resulted in phase II in only 1 of 24 injections).

Local Anesthetic Properties of Tetrodotoxin Alone
Groups of rats were injected with different concentrations of tetrodotoxin in 0.1-ml volumes. Local anesthesia resolved completely within a few hours, and there was no phase II from the tetrodotoxin block (fig. 1). The duration of thermal nociceptive block increased with increasing tetrodotoxin concentrations (table 2). The motor effects of tetrodotoxin are described subsequently.

Local Anesthetic Properties of the Combination of Capsaicin and Tetrodotoxin
Table 1 shows the durations of thermal nociceptive block resulting from injections of various concentrations of capsacin, with or without a fixed concentration of tetrodotoxin. Block duration from the combination was greater than the sum of the durations from the two drugs alone, in some cases by a factor greater than three. Potentiation was seen at capsacin concentrations as low as 99 μM (which had no effect on thermal nociception when used alone). When increasing tetrodotoxin was added to a fixed concentration of capsacin, the effect

Table 2. Duration of the Phase I for Thermal Nociception from TTX, with or without 990 μM Capsaicin

<table>
<thead>
<tr>
<th>TTX Alone</th>
<th>TTX (μM)</th>
<th>Duration (min)</th>
<th>n</th>
<th>TTX + 990 μM Capsaicin</th>
<th>TTX (μM)</th>
<th>Duration (min)</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 (0–0)</td>
<td>12</td>
<td></td>
<td>0</td>
<td>0 (0–0)</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0 (0–0)</td>
<td>6</td>
<td></td>
<td>30</td>
<td>31 (0–48)</td>
<td>18</td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>30</td>
<td>0 (0–24)</td>
<td>12</td>
<td></td>
<td>45</td>
<td>204 (140–284)</td>
<td>6</td>
<td></td>
<td>0.0043</td>
</tr>
<tr>
<td>45</td>
<td>0 (0–0)</td>
<td>6</td>
<td></td>
<td>60</td>
<td>138 (0–298)</td>
<td>12</td>
<td></td>
<td>0.012</td>
</tr>
<tr>
<td>60</td>
<td>0 (0–0)</td>
<td>18</td>
<td></td>
<td>90</td>
<td>289 (189–326)</td>
<td>12</td>
<td></td>
<td>0.0003</td>
</tr>
<tr>
<td>90</td>
<td>88 (24–118)</td>
<td>11</td>
<td></td>
<td>120</td>
<td>377 (342–421)</td>
<td>6</td>
<td></td>
<td>0.007</td>
</tr>
<tr>
<td>120</td>
<td>109 (82–147)</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Durations are medians with 25th and 75th percentiles. P values are the result of Mann-Whitney U tests comparing combinations of TTX + 990 μM capsacin with TTX alone. Since comparisons are single, P < 0.05 is considered significant.

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was also supraadditive (table 2). The addition of 990 µM capsaicin, which has little effect alone, potentiated tetrodotoxin at all tetrodotoxin concentrations greater than 30 µM.

We did further investigations of the interaction between tetrodotoxin and capsaicin by constructing an isobologram (fig. 2). First, we calculated the population EC₅₀s for each drug (defined as the doses at which 50% of rats achieved maximal thermal nociceptive block; i.e., a thermal latency of 12 s). Second, we administered fixed-ratio combinations of 0, 0.25, 0.5, and 0.75 times the EC₅₀s of each compound (e.g., 0.25 EC₅₀s of tetrodotoxin + 0.25 EC₅₀s of capsaicin). This allowed us to calculate the EC₅₀ of the combination, which was 0.28 EC₅₀s of both tetrodotoxin and capsaicin (95% confidence interval, 0.21 to 0.35 EC₅₀s). This value, when plotted on the isobologram (fig. 2), fell well below the dotted “line of additivity” connecting the EC₅₀s of the individual drugs. This signified that the combination is synergistic. (Additivity is shown when the EC₅₀ of the combination falls on that line, and a position above the line indicates antagonism.)

To ensure that the increased effectiveness of tetrodotoxin plus capsaicin was not simply an effect of the Tween 80 in the injectate of tetrodotoxin, we injected 45 µM (n = 6) and 90 µM tetrodotoxin (n = 5) with 0.3% Tween 80. The resulting durations of thermal nociceptive block were not different from those obtained in the absence of Tween (P = 0.49 and 0.89 respectively). Similarly, 38 µM and 19 µM tetrodotoxin were coinjected with the same concentrations of Tween that had been used in the tetrodotoxin-plus-capsaicin combinations (0.45 and 0.3%, respectively). These injections resulted in one of six maximal blocks (17%, n = 6) in the rats that were injected with 38 µM tetrodotoxin and no block (n = 6) in those that received 19 µM tetrodotoxin. Tween 80 alone had no measurable effect on thermal nociception or motor function when injected at concentrations of 0.1, 0.3, 1, and 3% (n = 6 each).

Coinjection of tetrodotoxin and capsaicin did not increase the incidence of phase II. No phase II blocks resulted from 12 injections of 90 µM tetrodotoxin plus 990 µM capsaicin, compared with 1 block of 24 injections with 990 µM tetrodotoxin alone; 90 µM tetrodotoxin plus 3.5 µM capsaicin had 1 phase II response of 12 injections, compared with 4 blocks of 24 injections with 3.5 µM capsaicin alone.

**Antagonism of the Capsaicin-Tetrodotoxin Synergism by Capsazepine**

We used the vanilloid receptor antagonist capsazepine to establish the role of the vanilloid receptor in the potentiation of tetrodotoxin by capsaicin. Groups of rats were injected with 90 µM tetrodotoxin plus 99 µM or 990 µM capsaicin in combination with either 30 µM or 300 µM capsazepine. Table 3 shows the results. At the lower concentration of capsaicin (99 µM), the synergism was counteracted by both 30 µM and 300 µM capsazepine. At 990 µM capsaicin, the synergism was counteracted by 300 µM but not by 30 µM capsazepine (although the duration of thermal nociceptive block from the latter group also was not statistically different from tetrodotoxin alone, possibly suggesting partial antagonism).

**Functional Specificity of Capsaicin, Tetrodotoxin, and the Tetrodotoxin-plus-capsaicin Combination**

We compared the duration of motor deficits and thermal nociceptive deficits resulting from doses of capsaicin, tetrodotoxin, and their combination. For this comparison, we selected doses that were at or bracketed...
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Table 3. Effect of Capsazepine on the Duration of Thermal Nociceptive Block of the Tetrodotoxin—Capsaicin Combination

<table>
<thead>
<tr>
<th>Tetrodotoxin (μM)</th>
<th>Capsaicin (μM)</th>
<th>Capsazepine (μM)</th>
<th>n</th>
<th>Duration (min)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>—</td>
<td>—</td>
<td>11</td>
<td>88 (24–118)</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>99</td>
<td>—</td>
<td>12</td>
<td>270 (115–344)</td>
<td>0.32, 0.024</td>
</tr>
<tr>
<td>90</td>
<td>99</td>
<td>30</td>
<td>12</td>
<td>123 (46–195)</td>
<td>0.18, &lt;0.0001</td>
</tr>
<tr>
<td>90</td>
<td>99</td>
<td>300</td>
<td>12</td>
<td>45 (45–50)</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>990</td>
<td>—</td>
<td>12</td>
<td>289 (189–326)</td>
<td>0.07, 0.57</td>
</tr>
<tr>
<td>90</td>
<td>990</td>
<td>30</td>
<td>12</td>
<td>218 (66–390)</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>990</td>
<td>300</td>
<td>12</td>
<td>60 (50–227)</td>
<td>0.7, 0.023</td>
</tr>
</tbody>
</table>

Durations are medians with 25th and 75th percentiles. P values are from Mann-Whitney U tests. The first P value compares the group to 90 μM TTX alone, the second to TTX + capsaicin without added capsazepine. P < 0.025 is considered statistically significant.

(one above and one below) the EC_{50} for thermal nociception. As described before, capsaicin itself resulted in a sensory-selective block at all concentrations except the highest one, in which there was transient motor dysfunction as a result of systemic toxicity. Nerve blockade from tetrodotoxin alone showed significant motor predominance (table 4), whereas the tetrodotoxin-plus-capsaicin combinations showed no predominance of one function compared with the other. The relation between durations of motor and thermal nociceptive blockade were similar at all other concentrations of these compounds. Although the addition of capsaicin to tetrodotoxin decreased the ratio of the duration of motor and sensory block, the absolute duration of motor block was increased. For example, the duration of motor block for 90 μM tetrodotoxin alone was 88 (24–118) min, whereas that of 90 μM tetrodotoxin plus 990 μM capsaicin was 336 (254–392) min (P = 0.0001).

Injection of Tetrodotoxin during Phase II

We evaluated capsaicin's potentiation of tetrodotoxin to determine whether it could be attributed to the same mechanism that caused phase II by injecting 90 μM tetrodotoxin at the sciatic nerves of eight rats that had been injected with 9.9 μM capsaicin 24 h previously and had developed phase II. Thermal latency before injection with tetrodotoxin was 10.3 s (6.7–12 s); there was no detectable deficit in motor function. Consequently we used motor rather than nociceptive measures in this experiment. After injection of tetrodotoxin, motor block lasted 49 (47–53) min, which was less (P = 0.026) than that from 90 μM tetrodotoxin when injected into pristine rats (88; 24–118 min), and did not mirror the potentiation by coinjected capsaicin as described before.

Interactions of Other Vanilloid and Site 1 Sodium Channel Blocking Compounds

To show that tetrodotoxin potentiation is a general property of vanilloid compounds, we performed similar experiments with resiniferatoxin (table 5). Similar to capsaicin, resiniferatoxin resulted in phase I and II increases in thermal analgesia without a measurable effect on motor function, and it prolonged the thermal nociceptive block by tetrodotoxin. Phase II was seen only with 16 μM resiniferatoxin, occurring in five of six rats when injected alone and in one of five rats when injected with tetrodotoxin.

We verified that synergism was a general property of toxins acting at site 1 of the sodium channel by testing the interaction between saxitoxin and capsaicin. Ther-

Table 4. Comparison of Duration of Thermal Nociception or Motor Blockade from Capsaicin, TTX, or Both in Combination

<table>
<thead>
<tr>
<th>Compound or Combination</th>
<th>Duration of Thermal Nociceptive Block (min)</th>
<th>Duration of Motor Block (min)</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,300 μM capsaicin</td>
<td>52 (11–77)</td>
<td>0</td>
<td>18</td>
<td>—</td>
</tr>
<tr>
<td>60 μM TTX</td>
<td>0 (0–0)</td>
<td>0 (0–100)</td>
<td>18</td>
<td>0.028</td>
</tr>
<tr>
<td>90 μM TTX</td>
<td>88 (24–118)</td>
<td>153 (67–172)</td>
<td>11</td>
<td>0.011</td>
</tr>
<tr>
<td>19 μM TTX + 736 μM capsaicin</td>
<td>36 (0–69)</td>
<td>18 (0–65)</td>
<td>12</td>
<td>0.87</td>
</tr>
<tr>
<td>38 μM TTX + 1,472 μM capsaicin</td>
<td>163 (129)</td>
<td>161 (104–293)</td>
<td>12</td>
<td>0.51</td>
</tr>
</tbody>
</table>

Concentration of compounds were selected to be near the EC_{50} for thermal latency. Nineteen μM TTX + 736 μM capsaicin is the EC_{50} of the combination, 38 μM TTX + 1,472 μM capsaicin is the EC_{50} of the combination. Durations are medians with 25th and 75th percentiles; P values are the result of paired Mann-Whitney U tests comparing thermal nociceptive and motor block within each group. Since comparisons are single, P < 0.05 is considered significant.

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Table 5. Duration of Thermal Nociception from Resiniferatoxin, with or without 120 µM TTX

<table>
<thead>
<tr>
<th>Resiniferatoxin (µM)</th>
<th>Duration (min)</th>
<th>n</th>
<th>Resiniferatoxin + 120 µM TTX</th>
<th>Duration (min)</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 (0–0)</td>
<td>12</td>
<td>109 (82–147)</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.016</td>
<td>0 (0–0)</td>
<td>12</td>
<td>210 (159–309)</td>
<td>6</td>
<td>0.047</td>
<td></td>
</tr>
<tr>
<td>0.16</td>
<td>0 (0–0)</td>
<td>12</td>
<td>271 (247–314)</td>
<td>11</td>
<td></td>
<td>0.024</td>
</tr>
<tr>
<td>1.6</td>
<td>0 (0–0)</td>
<td>12</td>
<td>396 (373–449)</td>
<td>12</td>
<td></td>
<td>0.0004</td>
</tr>
<tr>
<td>16.0</td>
<td>444 (441–490)</td>
<td>6</td>
<td>510 (472–568)</td>
<td>5</td>
<td>0.29</td>
<td></td>
</tr>
</tbody>
</table>

Durations are medians with 25th and 75th percentiles. P values are the result of Mann-Whitney U tests comparing combinations of resiniferatoxin + 120 µM TTX with 120 µM TTX alone. Since there are four comparisons (to 120 µM TTX), P < 0.0125 is considered significant.

Table 6. Effect of Capsaicin on the Duration of Block by Bupivacaine

<table>
<thead>
<tr>
<th>Compound or Combination</th>
<th>Duration of Thermal Nociceptive Block (min)</th>
<th>Duration of Motor Block (min)</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.9 µM bupivacaine</td>
<td>91 (52–94)</td>
<td>86 (73–97)</td>
<td>12</td>
<td>0.24</td>
</tr>
<tr>
<td>3.9 µM bupivacaine + 990 µM capsaicin</td>
<td>89 (75–104)</td>
<td>72 (46–76)</td>
<td>11</td>
<td>0.007</td>
</tr>
<tr>
<td>P</td>
<td>0.57</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.7 µM bupivacaine</td>
<td>103 (92–126)</td>
<td>114 (92–157)</td>
<td>12</td>
<td>0.13</td>
</tr>
<tr>
<td>7.7 µM bupivacaine + 990 µM capsaicin</td>
<td>131 (120–189)</td>
<td>94 (90–134)</td>
<td>12</td>
<td>0.004</td>
</tr>
<tr>
<td>P</td>
<td>0.013</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Durations of block are medians with 25th and 75th percentiles. The P values in the last column are from paired Mann-Whitney U tests comparing the durations of sensory and motor block. The P values in the third and sixth rows are from unpaired Mann-Whitney U tests comparing block durations.

Discussion

We have shown a synergism between tetrodotoxin and capsaicin in sciatic nerve blockade. Capsaicin increased the duration of thermal analgesia by tetrodotoxin in a supraadditive manner and reduced the dose of tetrodotoxin needed to achieve maximal block of thermal nociception. The supraadditive prolongation of the duration of nociceptive block by tetrodotoxin plus capsaicin was mediated by the vanilloid receptor, as shown by its nullification by capsazepine.

The theoretical basis for these experiments, as outlined in the introduction, is that capsaicin and tetrodotoxin potentiate each other because they inhibit different populations of sodium currents (mediated by tetrodotoxin-resistant and tetrodotoxin-sensitive channels, respectively). However, other explanations are possible for the observed effect. Capsaicin might decrease nerve blood flow and so potentiate tetrodotoxin in a
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manner analogous to that described for epinephrine. Studies have documented that capsaicin causes vasoconstriction, vasodilation, or both, depending on the dose, modes of administration, species, and study paradigm. Application of 1 to 10 μm capsaicin to mammalian pial vessels induces vasoconstriction that lasts approximately 1 min, followed by a longer period of vasodilation. The epineural application of capsaicin to the sciatic nerve has been shown to increase endoneural blood flow by 80% and to decrease endoneurial microvascular resistance by 45% within 3 min of injection. Vasoconstriction is probably not the mechanism of potentiation because (1) capsaicin dilates the vasa nervorum and (2) the vasodilatation that capsaicin induces in other vascular beds is very transient and unlikely to have much effect on the local kinetics of tetrodotoxin. Alternatively, capsaisin may increase nerve permeability and enhance tetrodotoxin entry into nerves. However, although capsaisin has been shown to affect nerve ultrastructure and function within the time frame of phase I, no data exist to suggest that it increases the permeability of neural tissue. Blood-brain barrier permeability is not increased by capsaisin at concentrations that increase vascular permeability in peripheral tissues. Furthermore, if capsaisin was simply potentiatating tetrodotoxin by increasing its penetration of neural tissue, we would expect blocks resulting from the combination to have the characteristics of tetrodotoxin, whereas our results showed that the combination is different (equal duration of sensory and motor function). Finally, the potentiation of tetrodotoxin could be caused by a toxic effect of capsaisin on nerve related to phase II. But this is unlikely because our data show that tetrodotoxin block is not potentiated by capsaisin at the time of phase II. More definitive treatment of these issues is beyond the scope of these experiments and may require studies of nerve blood flow and of tetrodotoxin flux into the nerve in the presence and absence of capsaisin.

We have shown a biphasic effect of capsaisin on thermal nociception, with phase I having rapid onset and waning within hours, and phase II having an onset of various hours after injection and lasting for days. Both phases affected thermal nociception and not motor function. Phase II probably corresponds to the effects described by Jancso et al. and Fitzgerald and Woolf after the direct application of capsaisin to rat sciatic nerve. Phase I has not been reported but may correspond to the conduction blockade that has been described in vitro. (Interestingly, a phenomenon similar to phase I has been described in rodents given single systemic doses of capsaicin, but involving an elevation in the nociceptive paw pressure threshold with no change in hot plate latency.) We postulate that phase I is mediated by blockade of sodium currents, and phase II may be caused by the ablation of C-fiber polymodal nociceptors. Another explanation might be depletion of substance P in the spinal cord, which also has been shown to follow a biphasic time course after the systemic administration of capsaicin. The time course of substance P depletion did not correlate with that of thermal analgesia in that report, but the investigators did not detect a biphasic thermal analgesia response. An alternative hypothesis would be that vanilloids enhance the synthesis of inhibitory neurotransmitters in the spinal cord. The problem with these explanations is that they would require capsaisin to reach the spinal cord from the injection site at the greater trochanter. Although injected capsaicin is rapidly distributed, the amounts that are delivered to remote neural tissues when capsaicin is administered perineurally are not high enough to have an effect, particularly at the very small doses we have given (990 μm capsaicin corresponds to approximately 86 μg/kg).

Phase II occurred with a frequency of 1 in 24 (4.2%) in rats injected with 990 μm capsaicin and in none of 12 rats injected with tetrodotoxin and 990 μm capsaicin. No phase II was seen in any rat injected with 550 μm capsaicin, with (n = 12) or without (n = 12) tetrodotoxin. Nevertheless, appropriate studies need to be done to determine whether these formulations can be used with a clinically acceptable risk of neurotoxicity. The development of less neurotoxic vanilloids may increase the margin of safety of such combinations.

Phase I may have been missed in previous investigators because they used much higher concentrations of capsaicin and because of the choice or concentration of solvent. We selected Tween 80, an agent that is approved by the Food and Drug Administration for injection, rather than solvents with known neurolytic potential (e.g., ethanol). The concentration of solubilizing agent was also minimized (some previous investigators used 10% Tween in paraffin oil, or 10% ethanol, or both). The importance of these factors is shown by the fact that we also could not discriminate phase I (because it was continuous with phase II) when we injected rats with 9.9 μm capsaicin in 3% Tween 80.

The coincidence of capsaicin altered the functional specificity of tetrodotoxin so that the combination was less motor selective. Nonetheless, the motor block by tetrodotoxin was prolonged by capsaicin. This was
somewhat unexpected because capsaicin is not believed to have acute effects on Aδ-fiber or Aβ-fiber function. It is conceivable that it could modify activity in small diameter Aδ axons are essential to the integrity of feedback loops that control muscle tone.

Capsaicin also showed synergism with saxitoxin, another site 1 sodium channel blocker with many mechanistic similarities to tetrodotoxin.11–13 Similarly, there was synergism between resiniferatoxin and tetrodotoxin. Thus, synergism appears to be a general property of site 1 sodium channel blockers and vanilloid receptor agonists. Resiniferatoxin51 is reported to be 3 to 10,000 times more potent than capsaicin.52 In our experiments, the potentiation of tetrodotoxin occurred at a capsaicin concentration of approximately 99 μM, and, with resiniferatoxin, the concentration was at least as low as 1.6 μM. The durations of thermal nociceptive block from tetrodotoxin plus 0.016 and 0.16 μM resiniferatoxin were much longer than that of tetrodotoxin alone, but the P values for their comparison to tetrodotoxin alone were not significant by the very conservative statistical method we have used. Thus, resiniferatoxin was at least 62 times more potent than capsaicin in terms of the concentration at which the duration of block of tetrodotoxin was potentiated.

Although bupivacaine is also a sodium channel blocker, capsaicin did not increase the duration of thermal nociceptive block of 3.9 mEq bupivacaine in phase 1, and it caused only a modest potentiation of the duration of block of 7.7 mEq bupivacaine. This was unlike the marked synergism seen with tetrodotoxin. The bupivacaine–capsaicin potentiation could be much weaker because conventional local anesthetics can affect both tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels.52,53 Alternatively, if the potentiation of tetrodotoxin (which is very hydrophilic) by capsaicin were a result of an enhancement of tetrodotoxin flux through the perineurium secondary to capsaicin's hydrophobicity, then we might expect that bupivacaine (which is more hydrophobic than tetrodotoxin) would not derive any such benefit. We do not consider the latter speculation to be likely, as noted before. It is also possible that the mild prolongation of the duration of thermal nociceptive block is caused by vasocostriction from bupivacaine54 by delaying clearance of capsaicin from the site of injection, in a manner analogous to that in which we have speculated that bupivacaine may affect tetrodotoxin.28 However, if that were the case, we might expect motor blockade also to be prolonged. Bupivacaine greatly increased the incidence of phase II. This effect might arise from a synergism between the local neurotoxicity of amino amide anesthetics55 and capsaicin. It might also be caused by an exaggeration of capsaicin neurotoxicity, because of the vasoconstrictive effect of bupivacaine in maintaining a high local concentration of capsaicin. In comparison, tetrodotoxin did not increase the incidence of phase II. This is consistent with tetrodotoxin's known lack of local neurotoxicity29 or vasoconstrictive properties.56

The EC50 for tetrodotoxin in this study was 76 μM. This is twice the EC50 that we reported recently28 (37.6 μM). This difference can be attributed to the fact we used a larger volume of injectate (0.3 ml) in the previous report. The current formulation is more efficient in that the EC50 is achieved by 2.5 μg tetrodotoxin, compared with 3.8 μg in the more dilute formulation. As a result, longer blocks were achieved for a given dose and associated toxicity.

In conclusion, vanilloid compounds and site 1 sodium channel blockers have a synergistic interaction in peripheral nerve block, mediated by the vanilloid receptor. This potentiation lends support to the hypothesis that simultaneous inhibition of tetrodotoxin-sensitive and tetrodotoxin-resistant sodium currents has a synergistic effect on functional local anesthesia. These interactions may provide clues for designing local anesthetics with prolonged durations. Vanilloid compounds may help elucidate mechanisms of sensory selective nerve blockade by virtue of their ability to alter the functional specificity of tetrodotoxin.

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