The Local Anesthetic n-Butyl-p-Aminobenzoate Selectively Affects Inactivation of Fast Sodium Currents in Cultured Rat Sensory Neurons

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Background: Aqueous suspensions of the local anesthetic n-butyl-p-aminobenzoate (BAB), epidurally applied in terminal cancer patients, resulted in a sensory blockade, lasting up to several months. To investigate the mechanism of action on the cellular level, the effect of 100 μM BAB on Na⁺ action potentials and on Na⁺ currents in dorsal root ganglion neurons from neonatal rats was studied.

Methods: Small neurons grown in cell culture were selected for patch-clamp measurements. Both Na⁺ action potentials, evoked by current pulses of increasing amplitude (current clamp) and Na⁺ currents, activated at different membrane potentials (voltage clamp), were investigated in the absence and presence of 100 μM BAB. The local anesthetic was applied by external perfusion for 2 or 10 min.

Results: In the presence of 100 μM BAB, either the firing threshold was raised or the action potential was abolished. The maximal peak conductances, underlying the fast sodium current I₅Na and the slow sodium current IₓNa, were not changed. However, the inactivation of I₅Na, was increased by BAB. The sigmoid inactivation curve shifted 12 mV toward hyperpolarizing membrane voltages, whereas no changes were found for the inactivation of the slow Na⁺ current. Only at short exposure times of 2 min, the effects of BAB could be reversed during a 10-min wash-out.

Conclusions: BAB dramatically increased the firing threshold, and in part of the sensory neurons, it blocked the action potential. The inactivation of the fast Na⁺ channels, but not of the slow Na⁺ channels, was increased by BAB. Thus, the block of fast Na⁺ channels by BAB may contribute to epidural analgesia. At exposure times of 10 min, the effect of BAB was not reversible. This probably originates from its high lipid-solubility, which may be an important factor in determining the duration of the block in vivo. (Key words: Anesthetic, local; n-butyl-p-aminobenzoate; Ions; Na⁺. Measurement technique: patch-clamp. Na⁺ current inactivation. Nerve: dorsal root ganglion.)

PAIN treatment in terminal cancer patients often involves application of opioids and local anesthetics or sometimes lesions of sensory nerves by means of alcohol or phenol. These treatments may cause severe side effects, among which motor dysfunction is most prominent. An aqueous suspension of the highly lipid-soluble local anesthetic n-butyl-p-aminobenzoate (BAB) has been injected epidurally on the segmental level of pain perception in terminal cancer patients. The resulting depot of BAB is believed to dissolve slowly in the epidural space, thus causing a gradual and continuous application of BAB. This treatment resulted in a long-lasting (median 29 days) sensory blockade (segmental analgesia), combined with a marked reduction of pain, without a reduced motor function. The use of such a suspension is a promising alternative for the classic methods of pain treatment in terminally ill patients.

The preservation of motor functions during and after BAB treatment suggests that the epidural application of the local anesthetic has a selective effect on sensory nerve fibers. It has been proposed that selective block after application of local anesthetics originated from differences in internodal distance of sensory and motor fibers, in relation to the length of nerve exposed to the local anesthetic. Korsten et al. postulated that the selective block seen after epidural BAB is caused by exposure of spinal roots crossing the epidural space. This selective effect is not nullified by diffusion to the cerebrospinal fluid, because BAB is hydrophobic.
Therefore, the length of nerve exposed to BAB is limited and may explain the selective block of sensory fibers. To investigate the mechanism of action of BAB on a cellular level, the patch-clamp technique was applied to isolated dorsal root ganglion (DRG) neurons from the rat. These cells are the primary sensory neurons of higher vertebrates, a subpopulation of which mediates pain perception. The DRG neurons are close to the site, where the remnant of the BAB suspension was found in humans at necropsy. In the current study, a primary culture of DRG neurons from newborn rats was used, and attention was focused on the sodium currents in these cells. Sodium currents, in general, are important contributors to excitation in most types of neurons and, therefore, form an excellent target for substances inhibiting signal propagation. Without excluding the possibility that BAB mediates its effects via the modulation of other ion currents, e.g., K+ or Ca2+, Na+ currents were deemed the most beneficial candidates for a first investigation.

The current study primarily addresses the question of whether the two types of sodium current present in DRG neurons, are influenced by application of BAB and, if so, in which way. The properties studied are the peak Na+ conductance underlying the fast and slow Na+ currents and the steady-state inactivation of these currents. A part of the results has been described in abstract form.

Materials and Methods

Neonatal rats 1 day of age were killed by decapitation, and the DRGs from different levels of the spinal cord were rapidly collected under sterile conditions. With a fine forceps, three or four ganglia were brought into a drop of culture medium on circular cover glasses coated with poly-D-lysine (Sigma) and placed in culture dishes. The ganglia were mechanically dissociated with the fine forceps, and the neurons were allowed to attach to the coated glass for 4 h in a humidified 5% CO2 atmosphere at 37°C. Thereafter, we added approximately 1 ml chemically defined culture medium supplemented with nerve growth factor. Within 24 h, the cells were used for patch clamp measurements. To this end, the cover slip was mounted in a chamber on the stage of an inverted microscope (Zeiss IM 35). In the patch pipette holder electrical contact between amplifier and pipette solution was made by an Ag-AgCl electrode. Patch-pipettes, pulled on a vertical two-stage electrode-puller, were from borosilicate glass (Clark GC-150 TF) and had tip diameters of approximately 3 μm. Their resistances varied between 1 and 4 MΩ.

Under current clamp conditions, 10% of the DRG neurons, cultured under our circumstances, exhibited Ca2+-action potentials. To exclude them, we used Ca2+-free extracellular solutions (ECS). The composition of the control ECS (control-ECS) solution was as follows (in mM): NaCl 130, choline-Cl 10, KCl 5, MgSO4·7H2O 2.5, Na2[H2N-(2-hydroxyethyl)ipiperazine-N′-2-ethanesulfonic acid] (HEPES) 10, and glucose 10. The second and third type of ECS had a similar composition but contained, respectively, BAB [100 nm (BAB-ECS)] of 200 nm of the fast Na+ channel blocker tetrodotoxin (TTX; TTX-ECS) and each 300 nm from the voltage-clamp conditions (VC) to compensate for the age EPC due to TTX pulses. In all conditions, the compensation of the TTX-applied current was made constant with (control-ECS) 5, HEPES 10, Na2ATP 2, and glucose 10. For measurement of Na+ currents under voltage-clamp (VC) conditions, the pipettes were filled with ICS, (in mM): CsOH 15, EGTA 5, MgSO4·7H2O 2.5, HEPES 10, CsCl 120, NaCl 8, tetrathylammonium-chloride (TEACI) 5, Na2ATP 2, and glucose 10. This type of ECS was used to isolate the Na+ currents, because Cs+ and TEA+ are inhibitors of the K+ current. Three types of Ecs were used. The composition of the first type (control-ECS) was in mM: NaCl 130, CsOH 5, HEPES 10, TEACI 10, MgSO4·7H2O 2.5, 4-AP amiloride (4-AP) 1, and glucose 10. Here too Cs+, TEA+, and 4-AP were used to reduce the K+ current as much as possible. The absence of external Ca2+ was necessary to block the Ca2+ current without influencing the sodium current. The second and third type of ECS had a similar composition as control-ECS, but included 200 nM TTX (TTX-ECS) and 100 μM BAB (BAB-ECS).

The ECS was produced in one large batch, whereas the intracellular solutions were prepared for the use of a few weeks at most. ATP and glucose were added to the ECS before the experiment. All solutions were stored at 5°C, and their pH was adjusted to 7.4 immediately before the experiment. The dish containing the cells was continuously perfused with ECS, at a rate of 2 ml/min. Electrical valves in the perfusion system allowed switching between ECS types of different ionic strength and temperature.

Currents were filtered (Bessel LPF, List EPC 960, CA) and stored digitally (List EPC 960, CA) on patch-pers. Data were analyzed in order to establish the current-clamp protocol, as a current of 100 pA above the threshold (10 Hz). The stimulation protocol was 3 pulses of 0.5 ms at 30 Hz from the pulse trigger. The cell was conditioned with no current to the age EPC due to TTX pulses compensation. In all conditions, the compensation of the TTX-applied current was made constant with TTX. The peak reversed potential, Vrev, was determined by placing a reversal voltage, Vrev, and measuring the current. The Na+ current, INa, was measured by subtracting the leak current from the total current I (excluding transient outward current). The data were fitted with the equation I = gNa(h - N(V - V1/2))exp(-A(V - V1/2))/(N - 1), where gNa was fit to a voltage of 0 mV to fit the peak current. The data were fitted with the equation I = gNa(exp(-(V - V1/2)/A)) + gK(exp(-(V - V1/2)/B)), where gNa was fit to a voltage of 0 mV to fit the peak current. The data were fitted with the equation I = gNa(exp(-(V - V1/2)/A)) + gK(exp(-(V - V1/2)/B)), where gNa was fit to a voltage of 0 mV to fit the peak current. The data were fitted with the equation I = gNa(exp(-(V - V1/2)/A)) + gK(exp(-(V - V1/2)/B)), where gNa was fit to a voltage of 0 mV to fit the peak current. The data were fitted with the equation I = gNa(exp(-(V - V1/2)/A)) + gK(exp(-(V - V1/2)/B)), where gNa was fit to a voltage of 0 mV to fit the peak current. The data were fitted with the equation I = gNa(exp(-(V - V1/2)/A)) + gK(exp(-(V - V1/2)/B)), where gNa was fit to a voltage of 0 mV to fit the peak current. The data were fitted with the equation I = gNa(exp(-(V - V1/2)/A)) + gK(exp(-(V - V1/2)/B)), where gNa was fit to a voltage of 0 mV to fit the peak current. The data were fitted with the equation I = gNa(exp(-(V - V1/2)/A)) + gK(exp(-(V - V1/2)/B)), where gNa was fit to a voltage of 0 mV to fit the peak current.
allowed quick changes (±20 s) between the different types of ECS. All experiments were performed at room temperature, which was maintained at 19°C.

Current clamp (CC) and voltage clamp (VC) protocols were provided by a custom-built pulse generator. The membrane voltages or currents, recorded by the List EPC-7 amplifier, were filtered at 10 kHz (4-pole Bessel), fed into an A/D-converter (Biomation), and stored on a PDP 11/73 minicomputer. The tip of the patch-pipette was sealed on the cell surface, and the patch membrane was ruptured by suction (10–40 kPa) to establish the whole cell clamp mode. Under current clamp conditions, we applied the threshold protocol, consisting of 10–20 depolarizing current pulses of 10 ms, with an amplitude increasing from subthreshold to suprathreshold levels at a frequency of 1 Hz. The membrane voltage, at which the action potential appeared for the first time, was taken as the firing threshold of the neuron. Supramaximally evoked action potentials were recorded each 6 s for 2 min or each 30 s for 10 min to follow the onset and recovery from the local anesthetic. Under voltage-clamp conditions, the neurons were clamped at the holding voltage $V_h$ of −70 mV. During hyperpolarizing voltage pulses to −140 mV, the capacity currents were compensated by adjusting the capacity cancellation settings of the amplifier. The access resistance to the cell interior was compensated for 50–80%. Readjustments of all compensations were necessary during the experiment. Residual capacity currents $I_c(t)$ were digitally corrected. The ionic leakage current $I_l$ of the neuron was measured in the voltage range from −80 to −140 mV. As expected, this current, flowing through the voltage-independent ion channels, was linear and could be described by:

$$I_l = g_l(V - E_l),$$

where $g_l$ is the leakage conductance and $E_l$ the leak reversal potential. The parameters in equation 1 were used to subtract $I_l$ from the membrane currents measured at the different voltages. The measurement of the Na⁺ current ($I_{Na}$) required a fast spatial control of the membrane voltage. Neurons cultured for 1 day had no or short axons (<10 μm), and thus a uniform potential could be expected. However, a large Na⁺ current in conjunction with the residual access resistance could be responsible for inadequate space clamp. Therefore, we selected the smaller neurons, having diameters from 10 to 15 μm, to reduce the magnitude of the inward Na⁺ current, which could be as high as 15 nA in larger cells. This selection limited the magnitude of $I_{Na}$ to about 5 nA. Only neurons in which the Na⁺ current activated smoothly and where no delayed action currents appeared were accepted for the analysis of the data.

To understand the voltage protocols and the terminology, following is a simplified scheme of the main states of sodium channels:[13]

closed $\rightarrow$ open $\rightarrow$ inactivated $\rightarrow$ closed. (2)

The transitions between the different states depend on membrane voltage. At hyperpolarization, Na⁺ channels are closed; whereas at depolarization, they open (activation) and become inactivated. The transition from the inactivated to the closed state, thus the removal of inactivation, occurs at hyperpolarization. Two types of voltage protocols, under control of the PDP 11/73, were used: (1) the activation protocol, consisting of a 40-ms prepulse to −140 mV to remove inactivation and an 8-ms test pulse to measure the Na⁺ current. The test pulse was increased from −100 to +100 mV in 10 mV steps at the repetition rate of 1 Hz; and (2) the inactivation protocol, consisting of a 40-ms prepulse, varying from −140 to 0 mV in 5-mV steps, to change the degree of inactivation and a subsequent test pulse to −10 mV. Both protocols were applied on the same neuron in control- and BAB-containing solutions after an incubation period of 10 or 2 min, if quick readjustments of the voltage-clamp allowed such a short measurement time.

Data were retrieved from the minicomputer to a PC-AT (Procom 386) for data analysis. Special customized software (Bioav) was used to correct the measured Na⁺ currents for the contribution of $I_l(t)$ and $I_c(t)$. Curve-fitting was performed using the graphics program FigP6.0 (Biosoft). Statistical testing (Wilcoxon’s signed-rank test) was performed with the program StatGraphics. The level of significance was chosen as 0.05. Data are given as mean ± SEM, with $n$ the number of investigated neurons.

Results

Firing Threshold of Sodium Action Potentials

Action potentials were evoked from neurons with a stable resting membrane potential that was more negative than −50 mV. Figure 1. shows the effect of 100 μM BAB on the excitability of 15 of 22 accepted neurons: an increase of the firing level $V_{th}$, and the disap-

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Fig. 1. Whole-cell current clamp recordings from a cultured dorsal root ganglion neuron with a resting membrane potential of −64 mV. (Inset) Applied current protocol. Current pulses differ by 20 pA. (Left) In control-ECS, 0, V_{th} was −26 mV, the peak of the action potential 41 mV, and t_{peak} 8.9 ms (after the onset of the pulse). (Middle) The anesthetic (BABA-ECS_{A}) was applied for about 90 s, after which the voltage recordings in BAB were made. V_{th} amounted to −11 mV, the peak of the action potential 15 mV, and t_{peak} 12.7 ms. Thus, BAB increased the firing threshold and decreased the speed of the rising phase of the Na⁺ action potential. (Right) The washing-out recordings were obtained 2 min after the replacement of BAB-ECS{A} by control-ECS{A}. The magnitude of V_{th} was −27 mV, the peak of the action potential 39 mV, and t_{peak} 8.5 ms.

The appearance of the fast rising phase of the action potential. The threshold voltage was raised from −29 ± 2 mV in control-ECS{A} to −18 ± 2 mV (n = 15) in BAB-ECS{A}, which were statistically different (P = 0.0001). Provided that the BAB-ECS{A} was applied for 2 min or less, these effects were reversible. After washing-out, the threshold voltage was −27 ± 2 mV, not statistically different from the control value (n = 15). The time to reach the peak of the action potential, t_{peak}, was significantly increased (P = 0.007) from 5.7 ± 0.9 ms to 7.7 ± 1.3 ms (n = 8). Its value became 6.1 ± 1.0 ms after washing-out BAB, which did not differ statistically from the control value. In 7 of 22 neurons, the action potential was blocked by a 2-min application of 100 μM BAB in a reversible way. The threshold voltage of −26 ± 3 mV, determined in control-ECS{A}, did not differ statistically from the one obtained after washing-out BAB. All investigated neurons exhibited a pronounced effect on their excitability in the presence of 100 μM BAB.

The DRG culture is a heterogeneous population of neurons, which contain varying proportions of fast and slow Na⁺ channels. To relate the BAB effects to the possible blockade of fast or slow Na⁺ channels, the toxin TTX was applied before BAB perfusion or directly after washing-out the local anesthetic. At a concentration of 200 nm, TTX selectively blocked the fast Na⁺ current in our cultured cells. For the first group of neurons (n = 15), the effect of TTX proved similar to that of BAB: The firing threshold and the value of t_{peak} were increased significantly, respectively, from −25 ± 2 mV to −18 ± 1 mV (P = 0.0028) and from 6 ± 1 ms to 8 ± 2 ms (P = 0.017) in a reversible way. This strongly suggested that BAB in a concentration of 100 μM inhibited fast Na⁺ channels and that the BAB-resistant action potential originated from activation of the slow Na⁺ channels. In the second group of neurons (n = 7), in which BAB blocked the action potential, TTX also reversibly blocked it, supporting the view that fast Na⁺ channels are blocked by BAB.

During perfusion with BAB or during its washing-out, we measured the supramaximally evoked action potential each 6 s and followed amplitude changes as function of time. The time needed for BAB to depress neuron's excitability, t_{reset}, amounted to 50 ± 5 s (n = 13). The recovery of the BAB effect in control-ECS was accomplished in a time t_{recovery} of 96 ± 7 s (n = 11), and two neurons needed 2–3 min to recover. This reversible action was only clear when the neurons were incubated in BAB for about 2 min. In eight neurons, incubation with BAB for 10 min did dramatically prolong the recovery time to 420 ± 92 s (n = 5) or did not recover within 10 min (n = 5). Thus, the washing-out of BAB depends on the exposure time.

Peak Sodium Conductance

Several minutes after the establishment of the whole-cell voltage clamp configuration, outward K⁺ currents were blocked by Cs⁺ and TEA⁺, diffusing from the pipette into the cell interior. Under these conditions, inward and outward Na⁺ currents could be recorded in response to a series of depolarizing test pulses (ac-
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The magnitude of $g_{\text{Na}}$ and $E_{\text{Na}}$ in control solutions was, respectively, 48 nS and 66 mV. In the presence of BAB, for 2 or 10 min, the values of $E_{\text{Na}}$ (+68 mV) and $g_{\text{Na}}$ (47 nS) were not significantly different from the values obtained in control solutions (table 1). After the 10-min wash-out, the values of $g_{\text{Na}}$ and of $E_{\text{Na}}$ were not statistically different from their values obtained under control conditions (table 1).

The fact that $g_{\text{Na}}$ was not affected by BAB seems to contradict the TTX-like effect of BAB on the firing threshold and action potential. However, during the standard activation protocol, the hyperpolarizing prepulse to −140 mV may remove BAB-induced inactivation of the Na⁺ current. To investigate the role of the conditioning prepulse, we applied the activation protocol without the hyperpolarizing prepulse. Figure 5B shows the current-voltage relation under this condition for a neuron that exhibited predominantly $I_{\text{Na,F}}$. The peak currents considerably decreased in the presence of BAB. In five neurons, in which $I_{\text{Na,F}}$ dominated, the magnitude of $g_{\text{Na}}$ in control was $5.0 \pm 1.0$ nS, whereas in BAB, it was reduced to $0.64 \pm 0.26$ nS. The ratio of $g_{\text{Na}}$ in control and in BAB-containing solutions was $0.21 \pm 0.01$. After applying the hyperpolarizing prepulse, $g_{\text{Na}}$ was $17 \pm 7$ nS in control and in the presence of BAB, and their ratio was $1.0 \pm 0.1$ (n = 5). Thus, we can conclude that BAB induced a strong inactivation of the fast Na⁺ channels, which could be removed by hyperpolarization. After a 2-min exposure with BAB, the reduction of the currents could be reversed by washing-

$$R_{\text{p,T}} = \frac{I_{\text{p,max}}}{(E - E_{\text{Na}})}$$

Fig. 2. Effect of BAB on the sodium currents, measured in response to four test potentials, as indicated, preceded by conditioning prepulse. (Left) Activation protocol. For clarity, 4 current traces of 21 recordings are shown. By definition, inward currents are negative. (Middle) Currents after a 10-min perfusion in BAB (BAB-ECS). (Right) Currents after washing-out BAB for 10 min (control-ECS).
out the anesthetic (fig. 4). The ratio of \( g_{\text{Na},T} \) after a 10-min wash-out and its control value was 0.70 ± 0.14 (n = 4), thus, for the most part, reversible within this period.

**Separation of Fast and Slow Na⁺ Currents**

To separate the Na⁺ current in its fast and slow components at the test voltage of −10 mV, we considered that the fast current was inactivated at prepulse potentials, whereas the slow current showed no inactivation. Applying our inactivation protocol, the Na⁺ currents were obtained at the test potential of −10 mV as a function of prepulse potential (fig. 5A). When the prepulse was varied from −120 to −50 mV, the amplitude of the fast peak changed from maximal to zero, because of the progressive inactivation of \( I_{\text{Na},S} \). In the range of −70 to −40 mV, the slow current hardly changed, indicating the lack of inactivation of \( I_{\text{Na},S} \). At prepulse voltages between −40 and 0 mV, the slow current gradually disappeared, indicating that it became inactivated at these voltages. At the prepulse level of about −50 mV, we obtained the noninactivated slow sodium current \( I_{\text{Na},S}^{-30}(t) \) during the test pulse. By subtracting this current from the Na⁺ current \( I_{\text{Na},T}^{-30}(t) \) at a certain prepulse potential \( E_{pp} \) according to

\[
\Delta I(t) = I_{\text{Na},T}^{-30}(t) - I_{\text{Na},S}^{-30}(t).
\]

we could obtain the fast Na⁺ current component as function of \( E_{pp} \), with \( E_{pp} \) more negative than −50 mV. Figure 5B shows this procedure with \( E_{pp} = −140 \) mV, demonstrating the isolation of the fast activating and inactivating \( I_{\text{Na},T,F} \), which reached its maximum amplitude at this prepulse voltage. From the data, we calculated \( g_{\text{Na},F} \) according to equation 3, which was found to be 26 nS (table 1). After 2- or 10-min BAB perfusion, \( g_{\text{Na},F} \) was 25 nS, not significantly different from the control value. Similarly, from the peak value of \( I_{\text{Na},S}^{-30}(t) \) and using equation 3, we calculated \( g_{\text{Na},S} \) in all cases as 26 nS in control-ECS and in BAB-ECS as 17 and 13 nS, respectively. This difference was also not significant (table 1). After

![Fig. 3. (A) The peak amplitude \( I_p \) of the (total) sodium current is plotted as function of test pulse potential: peak current-voltage relation. (Inset) Activation protocol (not all test pulses shown) with a hyperpolarizing prepulse. Regardless whether BAB is present in the bathing medium, the sodium current activated at about −40 to −30 mV and reached its maximum amplitude at about 0 mV. The current reversed at about +66 mV, close to the calculated Na⁺ Nernst potential of +60 mV. (B) The peak current-voltage relation without a hyperpolarizing prepulse. (Inset) Activation protocol (not all test pulses shown). This neuron, different from that in figure 3A, exhibited predominantly the fast sodium current, and the blocking effect of BAB is obvious.

![Fig. 4. BAB abolished the peak current at about −140 mV.](http://anesthesiology.pubs.asahq.org/pdfaccess.ashx?url=/data/journals/jasa/931301/ on 06/19/2017)

![Table 1. Sodium Reversal Potentials and Peak Conductances](http://anesthesiology.pubs.asahq.org/pdfaccess.ashx?url=/data/journals/jasa/931301/ on 06/19/2017)

<table>
<thead>
<tr>
<th>Control</th>
<th>BAB</th>
<th>Wash-out</th>
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<tbody>
<tr>
<td>( E_{\text{Na}} ) (mV)</td>
<td>66 ± 3</td>
<td>66 ± 3</td>
</tr>
<tr>
<td>( g_{\text{Na},T} ) (nS)</td>
<td>48 ± 6</td>
<td>47 ± 7</td>
</tr>
<tr>
<td>( g_{\text{Na},S} ) (nS)</td>
<td>26 ± 8</td>
<td>25 ± 8</td>
</tr>
<tr>
<td>( g_{\text{Na},F} ) (nS)</td>
<td>17 ± 5</td>
<td>13 ± 4</td>
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The quantities obtained at the two different perfusion times were from different neurons and they were not significantly different.

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![Graphs showing control, BAB, and wash-out conditions](image)

Fig. 4. Effect of BAB on the fast sodium currents, measured in response to four test potentials (as indicated), applied in the absence of a conditioning prepulse. (Inset) Voltage protocol. (Left) Control currents. (Middle) Currents in the presence of BAB at about 2 min after application. The ratio of \( I_{\text{max}} \) in BAB and control is 0.16. With preceding hyperpolarization, this ratio amounted to 0.91. (Right) Washing-out BAB for 10 min.

the wash-out period, the magnitude of \( g_{Na} \) and \( g_{Na} \) did not differ statistically from the control values.

**Inactivation of the Fast and Slow Na⁺ Currents**

Steady-state inactivation of \( I_{Na} \) and \( I_{Na} \) was studied by varying the level of \( E_{\text{prep}} \) (inactivation protocol). From figure 5A, it is apparent that, at voltages of about −120 mV, the peak of the fast component became maximal, indicating that, at these potentials, its inactivation was removed. Therefore, we limited the amplitude of the prepulse to −140 mV. The fast and slow \( Na⁺ \) currents were separated according to equation 4 as a function of prepulse voltage. From the peak amplitude of the \( Na⁺ \) current component \( I_{Na} \), using a certain prepulse and its maximum amplitude \( I_{Na} \), obtained with the largest hyperpolarization, we calculated the ratio \( I_{Na} / I_{Na} \). This ratio is equal to the Hodgkin and Huxley inactivation parameter \( h \). The relation between \( h \) and \( E_{\text{prep}} \) was fitted with a Boltzmann equation of the form 1:

\[
h = \frac{1}{1 + e^{(E_{\text{prep}} - E_{\text{50}})/k)}},
\]

where \( E_{\text{50}} \) is the midpoint potential (50% inactivation) and \( k \) the steepness of the curve (slope factor). An example of the steady-state inactivation curves of \( I_{Na} \) and \( I_{Na} \), before and after exposure of 10 min to BAB, is given in figure 6. The lines were obtained by fitting equation 5 to the experimental data, which yielded the parameters \( E_{\text{50}} \) and \( k \). Because we will compare these parameters after perfusion with BAB and after washing-out, we determined them in control solution and after a repeated application of control solution or after washing-out TTX. Thus we could follow their values at different times (t), varying from 5 to 30 min after the start of the experiment. The changes \( \Delta E_{\text{50}} \) and \( \Delta k \) (\( \Delta \) value at time t − initial value) were analyzed as function of time. Linear regression of pooled \( \Delta E_{\text{50}} \) data on time yielded a regression coefficient (rc) of −0.154 ± 0.043 (n = 10) mV/min, significantly deviating from zero (\( P = 0.002 \)). Therefore, during a period of 10 min, the value of \( E_{\text{50}} \) may shift spontaneously 1.5 mV to hyperpolarizing voltages. Changes in the value of \( E_{\text{50}} \) (rc = 0.011 mV/min) and in the slopes \( k \) (rc = 0.016 mV/min) and \( k \) (rc = 0.017 mV/min) were not statistically different from zero.

The value of the midpoint potentials of the fast current \( E_{\text{50}} \) obtained in control-ECS, was −88 ± 2 mV (n = 19, pooled data). After a 2- or 10-min exposure to 100 μM BAB, the magnitude of \( E_{\text{50}} \), became −100 ± 2 mV (n = 19), significantly different from its value in control solution (\( P = 0.0001 \)). After the wash-out period, the value of \( E_{\text{50}} \) of neurons exposed to BAB for 10 min remained −100 mV, not statistically different from the value obtained in BAB. However, when BAB was applied for 2 min, it reversed during wash-out to −90 mV (table 2A/B), statistically different from the value obtained in BAB (\( P = 0.0001 \)). Therefore, BAB increased the steady-state inactivation of the fast \( Na⁺ \) channels, and this action could be reversed within 10 min only after the short-lasting perfusion. The significant difference (\( P = 0.0012 \)) between wash-out and control (table 2B) may arise from an incomplete wash-out or from the spontaneous shift of the inactivation.
Fig. 5. (A) Voltage-dependent inactivation of the whole-cell sodium currents. (Inset) Inactivation protocol to study Na⁺ current inactivation during prepulses $E_{pp}$, varying between −140 and +5 mV in steps of 5 mV and using a test potential of −10 mV. For clarity, 10 traces from 30 recordings are shown. Following the Na⁺ currents from the lower (at −140 mV) to the upper trace (at −5 mV), it is clear that the fast sodium current initially is selectively inactivated, whereas the slow sodium current remains virtually unchanged. At more depolarized prepulse potentials, the slow sodium current also is inactivated. The remaining current at −5 mV is the residual leakage current of the neuron. (B) Separation of fast and slow sodium current from whole-cell currents. (Inset) Voltage protocol with indicated levels of the 40-ms prepulse, test pulse, and holding voltage. Total sodium current $I_{Na,T}$ was recorded after the prepulse to −140 mV, showing both fast and slow sodium currents. After the prepulse to −50 mV, at which the fast sodium current inactivated, the slow sodium current $I_{Na,S}$ could be recorded. By subtracting the slow sodium current from the total sodium current (equation 4) the fast sodium current $I_{Na,F}$ could be isolated. The difference current thus obtained resembles the fast sodium current as recorded from neurons showing that current type only (see fig. 4).

curve. The value of the midpoint potential of the slow current $E_{so,s}$ amounted to $-28 \pm 2$ mV ($n = 20$) in both control and BAB-containing solutions. In neurons surviving the BAB treatment, its value was $-29 \pm 2$ mV ($n = 13$) after washing-out BAB (table 2). The slope factor of the fast current $k_f$ was $11 \pm 1$ mV ($n = 19$) in control and $10 \pm 1$ mV ($n = 19$) in the presence of BAB. For the slow component, the slope factor $k_s$ was $4.9 \pm 0.4$ mV ($n = 20$) in both control and BAB-containing solutions. Neither slope factor changed significantly in the presence of BAB or after washing-out the local anesthetic (table 2).

Discussion

The important findings of our study with 100 µM BAB are (1) an 11-mV increase of the firing threshold or action potential blockade; (2) a 12 mV shift of the inactivation curve of the fast Na⁺ channels to hyperpolarizing membrane voltages; (3) at exposure times of 2 min, but not of 10 min, the shift of the inactivation curve of the fast Na⁺ channels was reversible within a 10-min wash-out; (4) no shift of the inactivation curve of the slow Na⁺ channels; and (5) no alteration of the maximum peak conductances underlying the fast and slow Na⁺ current.

The increase of the firing threshold and the increase of the latency to the peak of the action potential in BAB were similar to the action of TTX. Action potentials blocked by BAB were also abolished by TTX. These findings indicated that, under the current conditions, the fast Na⁺ channels are the primary target of the local anesthetic. Because the resting membrane potential, determined by K⁺ channels, was not altered by BAB (fig. 1), these channels are probably insensitive for the local anesthetic. The action of BAB on the Na⁺ channels was fast. Considering that the time needed to replace the bath solution is 20 s, the effect of BAB on firing is accomplished within 30 s, whereas it is reversed within 80 s after the short exposure time of 2 min. However, after 10 min of BAB perfusion, its effects on Na⁺ action potentials and Na⁺ currents were not reversible within 10 min in most of the investigated neurons. Stable whole-cell patch-clamp recordings can last for about 30 min, after which an unacceptable rundown of the fast and slow Na⁺ currents becomes evident. The results of our experiments were consistent with these findings.
BAB INCREASED INACTIVATION OF FAST NA⁺ CURRENTS IN RAT SENSORY NEURONS

Fig. 6. Steady-state inactivation curves of fast and slow sodium currents in the absence and presence of 100 μM BAB. Circles are the values of the inactivation parameter h_in. closed circles in control-ESN, open circles in BAB-ESN. The lines represent the fit of equation 5 to these data, yielding the following parameters: E_h0 = -86.6 ± 0.5 mV, E_h = -17.7 ± 0.4 mV, k_h = 10.8 ± 0.4 mV, k_i = 4.1 ± 0.3 mV (control), E_h0 = -95.7 ± 0.2 mV, E_h = -18.5 ± 0.4 mV, k_h = 9.6 ± 0.2 mV, and k_i = 4.4 ± 0.3 mV (BAB). The inactivation curve of the fast sodium current (h_in) shifted approximately 10 mV in the hyperpolarizing direction after bath application of BAB-ESN. BAB had no apparent effect on the steady-state inactivation curve of the slow sodium current (h_s).

Because of possible spontaneous shifts of the inactivation curve, the mean E_h0, change of 12 mV in the hyperpolarizing direction has to be regarded as an upper bound. The shift reflects an increase in the number of inactivated fast Na⁺ channels by BAB at a given membrane potential. Therefore, at the resting membrane potential, for example, the available number of Na⁺ channels is considerably reduced, which will cause a substantial reduction of membrane excitability. The rise of the firing threshold and action potential blockade in the investigated neurons are in agreement with this prediction. The relatively large increase of the firing threshold in vitro may result in a complete action potential block in vivo, where small propagated action currents may not be able to cross the raised firing threshold.\(^{19}\) The clinical observation that epidurally applied BAB has a long-lasting analgesic effect in patients with terminal cancer\(^{12}\) is in accordance with the observed decrease of excitability.

Shifts of Na⁺ inactivation curves have been found for various anesthetics. In frog myelinated axon, the ethyl-analogs (1 mm) of BAB, benzocaine, metacaine, and ethyl-o-amino benzoate, shifted the inactivation curves respectively 24, 19, and 17 mV to the hyperpolarizing direction.\(^{20}\) However, contrary to our findings, the maximum peak Na⁺ conductances in these studies were considerably reduced. In the same preparation, lidocaine (1 mm) shifted the midpoint potential 30 mV toward hyperpolarizing voltages, while affecting the maximum Na⁺ conductance.\(^{21}\) Composed to these anesthetics, BAB only caused a relatively small shift of the inactivation curve, without reducing the maximal Na⁺ conductance. This difference may originate from the tenfold lower concentration of BAB in our test solution or from a specific property. Concentration-dependent shifts of the midpoint potential have been found for carboxylic ester anesthetics in squid axon.\(^{22}\) Shifting the Na⁺ inactivation curve is a common feature of local anesthetics and antiepileptic agents.\(^{23}\) In addition to such shifts, frequency- or use-dependent effects are important. For instance, lidocaine (50–200 μM) produced the slow Na⁺ current in a marked use-dependent manner, contrary to its blocking effect on the fast Na⁺ channels.\(^{16}\) These effects depend on the recovery time from the blocked or inactivated Na⁺ channels. In this respect, it is notable that the recovery from the increased inactivation due to BAB, e.g., at the holding voltage, is completed within the duration of the prepulse of 40 ms, whereas lidocaine needs tens of seconds.\(^{21}\) The fast (partial) recovery from block at

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Table 2. Midpoint Potentials and Slopes of the $E_r$ versus Voltage Relation of Fast and Slow Na$^+$ Currents in the Absence and Presence of 100 μM BAB at Exposure Times of 10 min (A) and 2 min (B)

<table>
<thead>
<tr>
<th></th>
<th>Control (mV)</th>
<th>BAB (mV)</th>
<th>Wash-out (mV)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Exposure time: 10 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E_{r,0}$</td>
<td>$-89 \pm 3$</td>
<td>$-98 \pm 4^*$</td>
<td>$-100 \pm 2^*$</td>
<td>11</td>
</tr>
<tr>
<td>$E_{r,SE}$</td>
<td>$-65 \pm 3$</td>
<td>$28 \pm 3$</td>
<td>$28 \pm 2$</td>
<td>12</td>
</tr>
<tr>
<td>$k_r$</td>
<td>$13 \pm 2$</td>
<td>$12 \pm 2$</td>
<td>$12 \pm 2$</td>
<td>11</td>
</tr>
<tr>
<td>$k_S$</td>
<td>$4.9 \pm 0.5$</td>
<td>$4.8 \pm 0.5$</td>
<td>$5.1 \pm 0.5$</td>
<td>12</td>
</tr>
<tr>
<td>(B) Exposure time: 2 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E_{r,0}$</td>
<td>$-87 \pm 2$</td>
<td>$-102 \pm 2^*$</td>
<td>$-90 \pm 1^*$</td>
<td>8</td>
</tr>
<tr>
<td>$E_{r,SE}$</td>
<td>$-32 \pm 1$</td>
<td>$-29 \pm 2$</td>
<td>$-29 \pm 1$</td>
<td>8</td>
</tr>
<tr>
<td>$k_r$</td>
<td>$10 \pm 3$</td>
<td>$10 \pm 3$</td>
<td>$9 \pm 3$</td>
<td>8</td>
</tr>
<tr>
<td>$k_S$</td>
<td>$4.9 \pm 0.8$</td>
<td>$5.1 \pm 0.8$</td>
<td>$4.9 \pm 1.3$</td>
<td>8</td>
</tr>
</tbody>
</table>

The quantities A and B were obtained from different neurons. The corresponding values in A and B were not statistically different, with the exception of the washout values for $E_{r,0}$ ($P = 0.0016$).

* Statistically different from control values.

Hyperpolarization is similar to that of benzocaine, showing a recovery time constant of 27 ms at $-120$ mV. After hyperpolarizing prepulse, the Na$^+$ currents, measured at depolarization, seem to be unaltered by the local anesthetic. It is tempting to speculate that, during hyperpolarization, the fast channel is unblocked and the voltage-dependent binding of BAB during depolarization is too slow to affect it, whereas at holding potential channel block can reach a steady-state level. Such an interpretation favors the modulated receptor hypothesis. However, various kinetic analyses should be performed to discriminate between different possibilities.

In the current study, we selected small neurons (20 μm), which probably correspond to the slow conducting Aδ- and C-fibers. Thus, our results seem applicable to pain neurons. Results from C-fibers of the isolated sural nerve indicated that the ED$_{50}$ value for the magnitude of the compound action potential equals 50-100 μM BAB. Therefore, the experiments described here were conducted using a test solution containing 100 μM BAB. The local anesthetic, dissolved from the epidurally injected suspension, will diffuse into the surrounding target tissues, i.e., dorsal root neurons and myelinated and unmyelinated nerve fibers. A concentration of at least 100 μM can be expected at these sites from the maximum solubility of BAB in water, amounting to approximately 1,000 μM/L. Whether concentration gradients along the nerves in conjunction with length-dependent block play a role, is not known. The interesting question why BAB selectively affects sensory neurons remains to be investigated.

In the neonatal DRG neurons, cultured for about 1 day, we found two types of Na$^+$ current. Fast and slow Na$^+$ currents have been found in DRG neurons, and in newborn rat, a third type of Na$^+$ current the $I_{Na,FP}$ may be present. The maximum peak inward current of $I_{Na,F}$ occurs at potentials varying between $-29$ mV and $-12$ mV, whereas the maximum inward peak of $I_{Na,SE}$ occurs at potentials of about $-5$ mV. Our recordings agree with these findings. The $I_{Na,SE}$, which is faster than the $I_{Na,F}$, should reach its maximum at $-20$ mV, and its midpoint potential is reported to be $-15$ mV. However, the constant amplitude of the peak of the fast current at prepulses up to $-140$ mV excluded significant contributions of $I_{Na,FP}$ to the Na$^+$ currents that we were measuring. Under control conditions, our value of $E_{Na,F}$ of $-88$ mV is more negative than reported values of $-65$ mV and $-75$ mV. Conversely, the value for $E_{Na,SE}$ of $-28$ mV is in the reported range from $-23$ mV to $-43$ mV. The difference between our estimate of $E_{Na,F}$ and reported values may originate from differences in the composition of the saline solutions and culture media. Under physiologic conditions, the resting membrane potential of the cultured DRG neurons amounts to $-64$ mV, and the firing threshold of Na$^+$ action potentials was $-25$ mV. With the current midpoint potential, all fast Na$^+$ channels inactivated at the firing level, indicating that the value of $E_{Na,F}$ will be more positive than $-88$ mV under more physiologic circumstances.

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The results reported here concern isolated sensory neurons from neonatal rats at room temperature. Although this preparation differs from the adult humans with whom the clinical study was performed, both isolated neurons and intact humans share a substantial number of properties. The fact that the results from the clinical study and the current one on isolated neurons apparently agree may lead to further understanding of the mechanism of action of long-lasting selective segmental analgesia after epidural administration of a 10% BABA-solution.

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References
20. Koppenhofer E, Sommer RG, Frosch U: Effects of benzocaine and its isomers on the sodium permeability and steady state sodium inactivation in myelinated nerve, obtained by an improved dissection technique. Gen Physiol Biophys 6:209-222, 1987
30. Ogata N, Tatebayashi H: Comparison of two types of Na+ currents with low-voltage-activated T-type Ca2+ current in newborn rat dorsal root ganglia. Pflugers Arch 420:590-594, 1992