'Butamben, a specific local anesthetic and aspecific ion channel modulator'

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CHAPTER 3

BLOCK OF TOTAL AND N-TYPE CALCIUM CONDUCTANCE IN MOUSE SENSORY NEURONS BY THE LOCAL ANESTHETIC N-BUTYL-P-AMINOBENZOATE (BUTAMBEN)

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ABSTRACT

In order to contribute to the understanding of the mechanism underlying selective analgesia by epidural application of suspensions of the local anesthetic butamben (n-butyl-p-aminobenzoate, BAB), the effect of dissolved BAB on calcium channels in sensory neurons was investigated. Small-diameter dorsal root ganglion neurones from newborn mice were used to measure whole-cell barium or calcium currents through calcium channels upon voltage-clamp stimulation. BAB suppressed the voltage-step evoked barium current of these cells in a concentration dependent way with an IC_{50} of 207 ± 14 µM (n = 40). A similar concentration dependency was found for the pharmacologically isolated N-type component of the whole-cell barium current. The time constants of inactivation and deactivation of the N-type current became smaller in the presence of BAB suggesting that kinetic changes are involved in the inhibition of this current. BAB caused a similar inhibition of the total calcium current as well as its N-type component, when these currents were evoked by command potentials with the shape of an action potential. This inhibition of calcium currents by BAB should be considered in the search for the mechanism of selective analgesia by epidural suspensions of the local anesthetic.

INTRODUCTION

Treatments of chronic pain may cause severe side effects, among which motor dysfunction is most prominent. A relatively new and promising approach to chronic pain treatment is the epidural administration of an aqueous suspension of the local anesthetic n-butyl-p-aminobenzoate (BAB), also known as butamben (Shulman, 1987; Korsten et al., 1991; Shulman et al., 1998). Application of the BAB suspension to the spinal dura results in a long lasting (median 29 days) relief from pain, without impairing motor function. This indicates that the BAB suspension selectively inhibits pain signaling sensory neurons, but the mechanism of its specific analgesic action is still unknown.

The BAB molecule is an aminobenzoate, ester-linked to a butyl group. The structure is similar to that of other ester-linked local anesthetics, such as benzocaine and procaine, which profoundly affect sodium channels involved in impulse generation and transmission in neurons. Effects of BAB on sodium currents have previously been studied in small dorsal root ganglion (DRG) neurons (Van den Berg et al., 1995; Van den Berg et al., 1996). However, the widespread opinion that the action mechanism of local anesthetics is mediated by sodium channels alone is, particularly for epidural anesthesia, an 'unproven assumption' (Butterworth and Strichartz, 1990).

Recently, we have shown in DRG neurons an effect of BAB on potassium channels and Kv1.1 channels in particular, which could contribute to the analgesia caused by the BAB suspension (Beekwilder et al., 2003). Calcium channels also play an important role in action potential firing of sensory neurons. A variety of calcium channel subtypes is expressed in sensory neurons of rodents (Mintz et al., 1992; Diochot et al., 1995). In the rat, N-type calcium current comprises ~50% of the total calcium current and is involved in calcium entry during action potentials in small diameter DRG neurons (Scroggs and Fox, 1992a; Blair and Bean, 2002; Bell et al., 2004), which include the pain sensing neurons (Scroggs and Fox, 1992b).

In the present study, we addressed the question whether voltage activated calcium channels are affected by BAB. To this end, the patch-clamp technique in

whole-cell voltage-clamp configuration was applied to acutely isolated small-size DRG neurons from neonatal mice. We did find inhibitory effects of BAB on the whole-cell current through calcium channels, including its N-type component. The physiological significance of these findings is considered in the discussion.

METHODS

Cell culture

Neonatal mice were killed by decapitation, and dorsal root ganglia from all accessible levels of the spinal cord were rapidly collected (approved by the Animal Ethics Committee at the Leiden University Medical Center). Cells were mechanically dissociated from two or three ganglia and cultured on a circular glass cover slip as previously described (Beekwilder et al., 2003). Within 8 h of culture, spherical neurons with a diameter of ~20 µm were selected for patch-clamp measurements. At this stage neurite outgrowth was still negligible.

Electrophysiology

For voltage clamp experiments a cover slip with DRG cell culture was mounted in a chamber on the stage of an inverted microscope. Patch pipettes were pulled from borosilicate glass (Clark GC-150 TF-15) and had resistances of 2.0 to 2.5 MΩ measured in the standard bath solution. Sintered Ag/AgCl electrodes coupled the amplifier input leads to the solutions. To minimize offset caused by low Cl^- pipette solutions, the pipette holder (Buisman et al., 1990) contained a Cl^- rich solution at the Ag/AgCl electrode.

Giga-seals were made in a microbath of ~75 µl, continuously perfused (~300 $\mu l \cdot min^{-1}$) with the standard bath solution (in mM): NaCl 145, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, pH 7.4 (NaOH). The pipette solution contained (mM): Cs-methanesulfonate 103, $MgCl_2$ 4, HEPES 9, EGTA 9, (Mg)ATP 4, (tris)GTP 1, (tris)phosphocreatine 14, pH 7.4 (CsOH).

After establishment of the whole-cell configuration the barium current was recorded with voltage-step protocols during extracellular perfusion with (mM): TEA-Cl 160, HEPES 10, EGTA 0.1, $BaCl_2$ 5, pH 7.4 (TEA-OH). A PC running Clampex 7 (Axon Instruments, Foster City, CA) and a List EPC 7 amplifier provided voltage protocols. Up to 80-90% of the series resistance was compensated. The membrane currents were filtered at 3 kHz in general and at 10 kHz for tail current measurements. Control experiments with an equivalent RC-circuit of the whole-cell showed that current transients with time constants of >100 µs can be reliably measured at 10 kHz filter setting (3-pole Bessel) under our conditions. All currents were leak subtracted using the P/4 method. Membrane capacitance of the selected DRG neurons was 14 ± 3 pF (n = 55). Calcium currents during action-potential clamp were measured under constant perfusion with (mM): TEA-Cl 160, HEPES 10, $CaCl_2$ 2, pH 7.4 (TEA-OH). The pipette solution was the same as above in the step voltage-clamp conditions.

Pharmacology

BAB (OPG Farma, Utrecht, The Netherlands) was added to the extracellular solution from a stock of BAB in ethanol (1-500 mM). Final ethanol concentration never exceeded 0.1 %. Because BAB has low water solubility (<700 µM at room temperature, Merck Index 1989) and easily binds to plastic surfaces of the perfusion system, final BAB concentrations up to 500 µM were verified using absorption spectrophotometry (290 nm). ω-Conotoxin-GVIA (CnTx; Peptide Institute Inc., Osaka, Japan) was dissolved in distilled water and added with a final fully blocking concentration of 3.3 or 5 µM (Diochot et al., 1995; Scroggs and Fox, 1992a; Blair and Bean, 2002; Scroggs and Fox, 1992b).

Analysis and statistics

Normalized data were corrected for rundown in the presence of vehicle (0.1 % ethanol) at all potentials measured in control experiments (n = 8). For example, at test pulses of 0 mV an apparent linear barium current decline (rundown) of ~ 6 % in 5 min was observed. The concentration-inhibition data were fitted using the Hill equation: $I/I_o = (1 + ([BAB]/IC_{50})^n)^{-1}$, where the IC_{50} is the concentration at which the current is reduced by 50% and n is the Hill coefficient. Results are presented as mean ± standard deviation (M ± SD) for n cells, unless stated

otherwise, and compared using paired or independent t-tests with the level of significance (p) chosen as 0.05.

RESULTS

BAB blocks whole cell barium currents

In order to assess the effect of BAB on calcium channels in neonatal mouse DRG neurons, barium currents through these channels were elicited by a square test pulse to 0 mV from –80 mV. Application of 200 µM BAB resulted in a decline of the current amplitude, reaching a steady-state value after 2-3 minutes. In Figure 1A the steady-state effect of 200 µM BAB is shown for a representative cell. At this BAB concentration the reduction of the peak whole-cell barium current amounted to 49 ± 7 % (n = 8). During washing out of the drug, the inhibiting effect of BAB proved to be partly reversible, reaching 86 % of the amplitude of

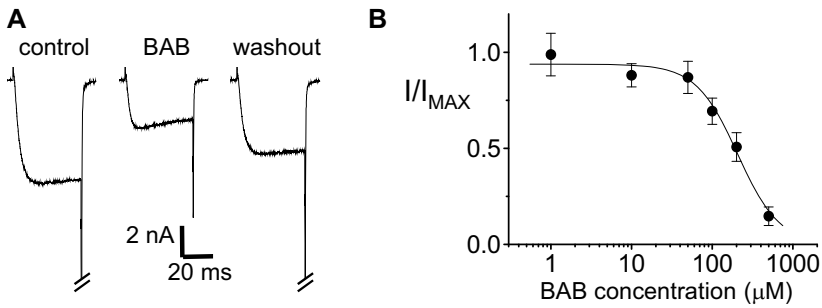

Figure 1: Effect of butamben (BAB) on whole-cell barium currents of a small neonatal mouse DRG-neuron. (A) Currents elicited by a test pulse to 0 mV from a holding potential of –80 mV at 0.1 Hz are shown for the control situation (left), after 3 minutes of 200 µM BAB exposure (middle) and after 3 minutes of washout (right). (B) Relationship between the applied BAB concentration and the relative peak amplitude of whole-cell barium currents (I/I_{MAX}) during test pulses of 0 mV, using an 80 ms prepulse to –120 mV from a holding potential of –80 mV. The solid line represents the fit of the Hill equation to the data. At each concentration n = 5–8 cells, with a total of n = 40. Data was corrected for rundown as described in the text.

the control situation (cf. Van den Berg et al., 1996).

The concentration-dependency of the current reduction by BAB was determined by measuring steady-state barium currents at different BAB concentrations. A prepulse to –120 mV was applied in order to remove possible inactivation at –80 mV (<10%). The peak currents in the presence of BAB were normalized to the corresponding whole-cell peak currents in the absence of BAB and were plotted as a function of concentration in the range from 1 to 500 µM (Figure 1B). A single Hill function could be fitted to the data yielding an IC_{50} of 207 ± 14 µM and a Hill coefficient of 1.7 ± 0.2 (n = 40).

BAB blocks N-type calcium channels

N-type calcium channels have a selective sensitivity to ω-conotoxin-GVIA (CnTx). In order to isolate the N-type current component, we used the procedure shown in Figure 2A,B. Whole-cell barium currents were measured in the absence and presence of 3.3 µM CnTx and the CnTx-insensitive currents were subtracted from the control currents (Figure 2B, left panel). CnTx caused an inhibition in peak current of 58 ± 5% (n=6). To investigate whether N-type currents are affected by the local anesthetic, currents were measured after preincubation with e.g. 200 µM BAB and after the subsequent perfusion with the CnTx solution still containing 200 µM BAB (Figure 2A, right panel). The resulting difference current represents the current through N-type channels in the presence of 200 µM BAB (Figure 2B, right panel). Repeating this procedure at different BAB concentrations and by plotting the normalized current density (pA/pF) as a function of BAB-concentration, the N-type concentration-response curve was obtained shown in Figure 2C (solid curve). Fitting the Hill equation to this relation yielded an IC_{50} of 220 ± 35 µM and a Hill coefficient of 1.4 ± 0.3 (n = 35).

The current decay of the N-type component during maintained depolarization (500 ms) was fitted with a single exponential function. The mean time constant in control solution was 78 ± 12 ms (n = 8), whereas in the presence of 200 µM BAB the N-type current inactivated significantly faster with a time constant of 64 ± 8 ms (n = 7, p=0.024).

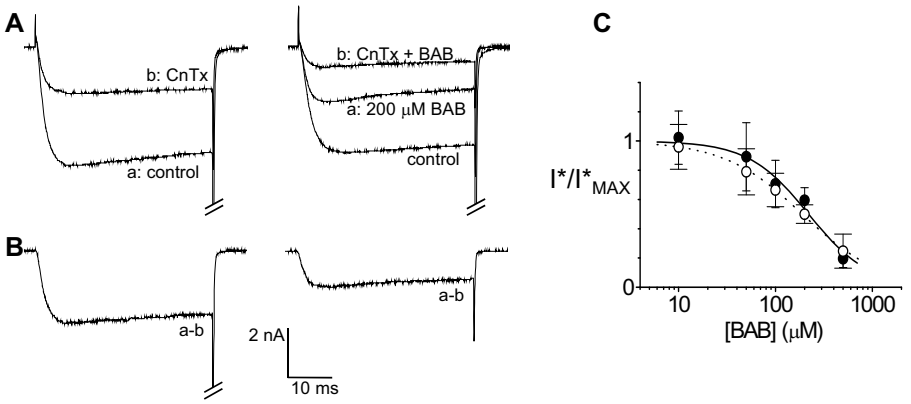

Figure 2: Effects of butamben (BAB) on CnTx-resistant and –sensitive barium current. (A) Control currents elicited by a pulse to 0 mV from a holding of –80 mV at 0.1 Hz with either 0 (left panel, trace a) or 200 μM BAB (right panel: trace a) followed by the application of 3.3 μM CnTx (ω-conotoxin-GVIA, traces b). In (B) the currents 'b' were subtracted from 'a', representing the CnTx-sensitive (N-type) current in the presence of either 0 (left panel) or 200 μM (right panel) BAB. In (C) the normalized current densities (I^*/I^*_{MAX}) of the CnTx-sensitive current (closed circles, solid line) and the CnTx-insensitive current (open circles, dotted line) are plotted against the applied BAB concentration. Current density (pA/pF) was used rather than current in order to minimize variability. At each concentration n = 5-8.

The tail current (cf. Figure 2B), representing the deactivation of the N-type channels, was elicited by stepping back from 0 to –80 mV and could also be fitted by an exponential function (fits not shown), yielding a time constant in control conditions of 167 ± 24 μs (n = 6). In the presence of 200 μM BAB the time constant was 136 ± 20 μs (n = 6), significantly lower (p=0.043) than that obtained under control conditions.

The residual current in the presence of CnTx represents the non-N-type current through calcium channels. It's BAB concentration-response curve is also given in Figure 2C (dotted curve) and is characterized by an IC_{50} of 189 ± 28 μM and a Hill coefficient of 1.1 ± 0.2. Because of its heterogeneity the non-N-type current was not further investigated.

BAB blocks action-potential clamp evoked whole-cell calcium currents

So far, we used voltage-steps to elicit barium currents. However, under physiological conditions calcium ions are the charge carriers and the calcium

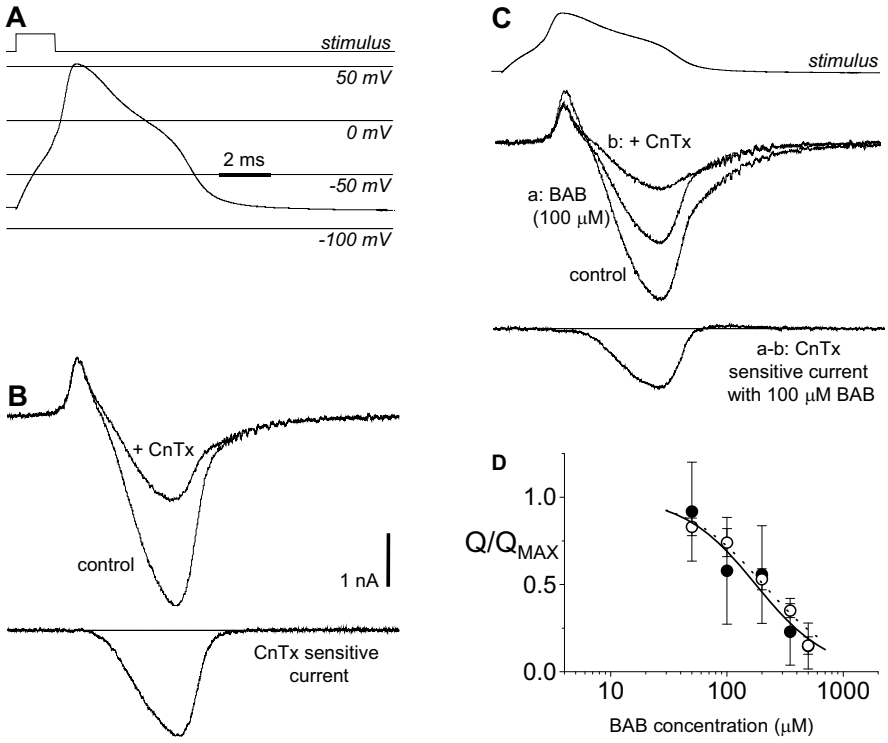

Figure 3: The effect of butamben (BAB) on calcium currents evoked by voltage-clamp stimulation with a 'standard' action potential at 6-s intervals. (A) Action potential recorded in current clamp in a small-size DRG neuron evoked by a 1.5 ms pulse of current injection. The action potential had a peak of +52 mV and a half width of 4.4 ms. Time scale bar applies to all panels. The cell was perfused with (in mM): NaCl 145, KCl 10, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, pH 7.4 (NaOH). The pipette solution contained: KCl 140, HEPES 10, EGTA 5, pH 7.4 (KOH). (B) Upper panel: Outward and inward currents under voltage clamp conditions elicited by the action potential from (A) as command voltage in the absence (control) and presence of 3.3μM CnTx (ω-conotoxin-GVIA) in a bath solution with 2mM calcium and 160mM TEA-Cl and with pipettes filled with Cs^+ as the main charge carrier (same pipette solution as in Figs. 1,2). Lower panel: The CnTx-sensitive current obtained by subtraction. Current calibration bar in (B) also applies to (C). (C) Upper panel: stimulus as in (A). Middle panel: Currents elicited by action-potential voltage-clamp stimulation under control conditions, in the presence of 100 μM BAB (record a) and in the additional presence of 3.3 μM CnTx (record b). Lower panel: The CnTx-sensitive current in the presence of 100 μM BAB, obtained by subtracting b from a. (D) The normalized integrated currents of the total calcium (open circles, dotted line) and the CnTx-sensitive current (closed circles, solid line) plotted against the applied BAB concentration. The lines represent fits with a Hill-equation. The n = 47 with at least 4 cells at each concentration.

channels are activated by naturally occurring changes in the transmembrane potential, e.g. during an action potential. Therefore, in the present study we also measured effects of BAB on calcium channels in the presence of a physiological concentration of calcium ions in the extracellular solution and using a previously recorded action potential as command voltage.

Figure 3A shows a representative action potential from a DRG neuron with a resting potential of around -75 mV. Although there is a marked neuron-to-neuron variability in action potential shapes, this action potential was applied as a standard voltage profile to get insight into the participation of the different calcium channels in the generation of the action potential. The 20-ms digitized standard action-potential record was applied from the holding voltage of –80 mV. The resulting control ion current is depicted in Fig. 3B and shows an initial outward current followed by an inward calcium current. This short outward current is resistant to application of 600 µM cadmium (blocking all inward current; n = 5) and does not interfere with the measurement of the subsequent inward calcium current. This current is likely carried by cesium ions flowing through unblocked (fast) sodium and potassium channels (Blair and Bean, 2002). The peak of the inward current coincided with the shoulder in the repolarizing phase of the action potential. The inward current decayed in two phases, an initial fast and subsequent slow one. The slower current decay occurred after nearly complete repolarization of the action potential, i.e. during the afterdepolarization.

BAB caused an overall decrease of the action potential clamp evoked calcium current (Fig. 3C). The initial positive current, the large negative peak and the fast and slow decay components were all affected. Figure 3D gives the concentration-response curve (dotted line) for the effect of BAB on the normalized integral (to reduce variability) of the total inward current. The parameters of the fitted Hill curve were an IC_{50} of 206 ± 8 µM and a Hill coefficient of 1.3 ± 0.1 (n = 47).

BAB blocks action potential clamp evoked N-type calcium currents.

During voltage clamping with the standard action potential, CnTx was perfused over the cell in order to specifically block the N-type current. CnTx did not affect the initial outward current, it neither affected the slower component of the

current decay, whereas the faster decaying current was removed to a great extent (Fig. 3B, upper panel). The difference between the total current and the current in the presence of CnTx is the N-type calcium current (Fig. 3B, lower panel). CnTx blocked 48 ± 10 % (n = 8) of the integrated current corresponding to the total calcium inflow. In order to determine the effect of BAB on the N-type component of the calcium current, action potential clamped currents were measured after preincubation with e.g. 100 μM BAB and after the subsequent perfusion with the CnTx solution still containing 100 μM BAB (Figure 3C, middle panel). The resulting difference current represents the current through N-type channels in the presence of 100 μM BAB (Figure 3C, lower panel). By repeating this procedure at different BAB concentrations, the concentration dependency of the BAB on the CnTx-sensitive current was determined. The relation of BAB and the normalized integral of the CnTx-sensitive current was described with a Hill equation (Figure 3D, solid curve), yielding an IC_{50} of 177 ± 47 μM and a Hill coefficient of 1.4 ± 0.5 (n = 47), similar to the parameters of the total calcium current (see above). For non-N-type calcium currents a similar concentration-response curve is implicated by the very similar curves in Fig. 3D.

DISCUSSION

The present study shows that the local anesthetic butamben (BAB) inhibits voltage clamp evoked barium and calcium currents including their N-type components.

Unlike sodium channels, where effects of 100 μM BAB ranged from a nearly complete block to insensitivity (Van den Berg et al., 1995; Van den Berg et al., 1996), calcium- and potassium channels show similarities in the measured effects of BAB. On both calcium (native) and Kv1.1 channels (native and cloned; Beekwilder et al., 2003) BAB caused an inhibition of the current with an IC_{50} of ~200 μM and a Hill coefficient of 1-2 and an accelerated deactivation. These data allow the possibility of two BAB binding sites per channel and suggest an allosteric mechanism of BAB action, by which the channel is biased towards the

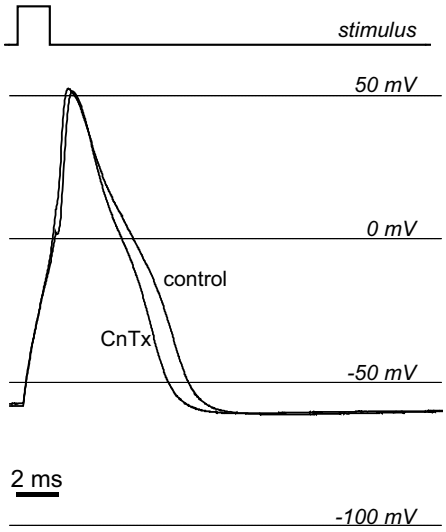

Figure 4: Action potentials evoked by current injections in the absence (control) and presence of 5 µM CnTx (ω-conotoxin-GVIA) , representative for 3 small DRG-neurons. The cell was perfused with (in mM): NaCl 145, KCl 10, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, pH 7.4 (NaOH). The pipette solution contained 140 KCl, 10 NaCl, 1 $CaCl_2$, 2 $MgCl_2$ 10 EGTA, 10 HEPES, pH 7.4 (KOH). Stimulus duration and amplitude were chosen to obtain just supramaximal stimulation with minimal interference of the evoked depolarization with the subsequent time course of the action potential. The slight delay in the onset of the action potential as well as the earlier repolarization in the presence of CnTx are illustrative for the role of N-type calcium channels in the excitability of these cells.

closed state. However, more experiments are needed to come to more definite conclusions about the mechanism of current reduction by BAB.

By using the action potential clamp and minimizing sodium and potassium currents total and N-type calcium currents flowing during a ′standard′ action potential could be measured . The shoulder of the action potential coincided with the peaks of the inward currents, as in rat sensory neurons (Scroggs and Fox, 1992a; Blair and Bean, 2002). The finding that CnTx eliminated about half of the total charge displaced through the calcium channels during the applied action potential, suggests a significant role for N-type calcium currents in nociceptive neurons. To illustrate the contribution of the N-type calcium current to the action potential waveform, Figure 4 shows the effect of CnTx on an action potential evoked in current clamp. Upon perfusion with CnTx the ′shoulder′ of

the action potential was partially removed, consistent with the results shown in Fig. 3 and results of others (Scroggs and Fox, 1992a; Blair and Bean, 2002). The importance of the N-type calcium channels in pain signaling is emphasized by findings that nociceptive neurons abundantly express a unique splice variant of the N-type channel (Bell et al., 2004) and that mice lacking the $Ca_v2.2$ gene, encoding for N-type calcium channels, show altered nociceptive responses (Hatakeyama et al., 2001; Kim et al., 2001; Saegusa et al., 2001). The present results indicate that non-N-type calcium currents are also inhibited by BAB. Inhibition of the low voltage-activated T-type calcium channels by BAB was confirmed in separate experiments (IC_{50} =178 ± 21 µM, Hill coefficient 1.5 ± 0.3, n = 40, for barium current peaks measured upon voltage steps from –80 to –40 mV). Thus, inhibition of both N-type and non-N-type calcium channels may contribute to BAB's epidural analgesic action.

The just below-maximal water-solubility BAB concentration of 500 µM (~2.5*IC_{50}) largely inhibited the total calcium current (Fig. 3D). The maximal-solubility concentration is the upper value in the clinical situation where BAB is applied as an aqueous suspension on the spinal dura. In the epidural space BAB diffuses from its depot and will affect the spinal nerves passing that space. Apparently, a local long-lasting BAB-concentration gradient is established as a result of the local balance between release, diffusion and degradation of BAB, including pharmacologically effective concentrations (Grouls et al., 1997).

The interesting question remains why epidural BAB-suspensions selectively affect the small diameter pain transmitting nerve fibres, while the thick motor and sensory fibres are not influenced. For the explanation of this differential blockade three mechanisms should be considered, which all may contribute. The first one explains differential nerve block with the classical observation that thinner axons cease firing with shorter segmental exposure to impulse blocking drugs than thicker axons (Franz and Perry, 1974). Korsten et al. (Korsten et al., 1991) explained in this way the selective action of BAB from differences in critical length of axons traversing the epidural space. Grouls et al. (1997) suggested that selective pain suppression by BAB was the result of a stable establishment of relatively low epidural concentrations due to the low solubility of BAB, which would favour inhibition of the thinner pain fibers. Finally, there are possible differences in BAB-sensitive ion channel expression in axonal

membranes of myelinated and unmyelinated fibers. A rich repertoire of sodium and potassium channels is present in Ranvier nodes, but calcium channels are lacking (Waxman and Ritchie, 1993). For the unmyelinated sensory fibres the spectrum of ion channels is not well studied, but apart from tetrodotoxin-sensitive and –resistant sodium channels, calcium channels belong to their ion channel palette (Elliott, 1990). Calcium spikes have been recorded from human nociceptive C fibers of the sural nerve (Quasthoff et al., 1995) and could be evoked by capsaicin (Mayer et al., 1999). It is thus tempting to speculate that calcium channels play a key role in selective analgesia by BAB by serving as targets for blocking the calcium spikes in pain transmitting fibers. In this respect it is of interest to mention that others have shown that some other, more hydrophilic, local anesthetics (e.g. bupivacaine) also inhibit calcium currents in mammalian sensory neurons (Sugiyama and Muteki, 1994) and dorsal horn neurons (e.g. ropivacaine) (Liu et al., 2001). Ropivacaine, which also has motor sparing properties, seems to act by another mechanism than BAB, since it must have a less localized epidural distribution because of its larger water solubility and insensibility to esterases (cf. Grouls et al., 1997) and because of its property to increase calcium currents at lower concentrations (Liu et al., 2001).

In conclusion, submaximal water-solubility BAB concentrations inhibit the calcium channels of sensory neurons. This inhibition is likely to contribute, in addition to the inhibition of sodium and potassium channels, to the long duration selective analgesia following epidural application of BAB suspensions.

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