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

Beekwilder, J.P.

CHAPTER 2

KV1.1 CHANNELS OF DORSAL ROOT GANGLION NEURONS ARE INHIBITED BY N-BUTYL-P-AMINOBENZOATE, A PROMISING ANESTHETIC FOR THE TREATMENT OF CHRONIC PAIN

J.P. Beekwilder, M.E. O'Leary, L.P. van den Broek, G.Th.H. Van Kempen, D.L. Ypey and R.J. Van den Berg.

J Pharmacol Exp Ther (2003) 304:531-8

ABSTRACT

In this study, we investigated the effects of the local anesthetic n-butyl-p-aminobenzoate (BAB) on the delayed rectifier potassium current of cultured dorsal root ganglion (DRG) neurons using the patch clamp technique. The majority of the K current of small DRG neurons rapidly activates and slowly inactivates at depolarized voltages. BAB inhibited the whole-cell K current of these neurons with an IC_{50} of 228 μM. Dendrotoxin K (DTX_K), a specific inhibitor of Kv1.1, reduced the DRG K current at +20 mV by 34%, consistent with an important contribution of channels incorporating the Kv1.1 subunit to the delayed rectifier current. To further investigate the mechanism of BAB inhibition, we examined its effect on Kv1.1 channels heterologously expressed in mammalian tsA201 cells. BAB inhibits the Kv1.1 channels with an IC_{50} of 238 μM, similar to what was observed for the native DRG current. BAB accelerates the opening and closing of Kv1.1, but does not alter the midpoint of steady state activation. BAB appears to inhibit Kv1.1 by stabilizing closed conformations of the channel. Co-expression with the Kvβ1 subunit induces rapid inactivation and reduces the BAB sensitivity of Kv1.1. Comparison of the heterologously expressed Kv1.1 and native DRG currents indicates that the Kv β1 subunit does not modulate the gating of the DTX_K-sensitive Kv1.1 channels of DRG neurons. Inhibition of the delayed rectifier current of these neurons may contribute to the long duration anesthesia attained during the epidural administration of BAB.

Epidural administration of local anesthetics is a widely used technique for achieving short-term regional anesthesia. A promising new approach for the management of chronic pain is the epidural administration of sustained release formulations of local anesthetics. For example, epidural injections of the local anesthetic n-butyl-p-aminobenzoate (BAB) has proved to be effective in treating the intractable pain associated with advanced stages of cancer (Korsten et al., 1991; Shulman et al., 1998). A single epidural treatment with BAB can effectively relieve chronic pain for prolonged intervals (>30 days). Surprisingly, the pain relief produced by BAB is not associated with any demonstrable loss of motor function suggesting that BAB selectively targets the nociceptive nerve fibers of the dorsal root (Korsten et al., 1991; Shulman et al., 1998; McCarthy et al., 2002). Because BAB is hydrophobic and uncharged at physiological pH, it partitions into lipid bilayers but does not effectively distribute into the systemic circulation (Kuroda et al., 2000; Shulman et al., 1998). The analgesia produced by BAB is highly localized with no detectable anesthesia in adjacent spinal segments (Korsten et al., 1991; Grouls et al., 2000). The absence of significant side effects coupled with the long duration anesthesia provides considerable support for the use of BAB formulations in the treatment of chronic pain.

The mechanism of BAB anesthesia and the origin of its highly selective block of nociception is not known. Studies of the mechanisms of BAB anesthesia have focused on small dorsal root ganglion (DRG) neurons as the most likely site of BAB action. In patch-clamp studies, BAB was found to inhibit the voltage-gated sodium currents of these neurons (Van den Berg et al., 1995; Van den Berg et al., 1996) which are believed to include the cell bodies of pain fibers (cf. Harper and Lawson, 1985). Small DRG neurons express several distinct components of Na current that differ in gating kinetics and sensitivity to tetrodotoxin (TTX) (Kostyuk et al., 1981; Roy and Narahashi, 1992). The TTX-sensitive and TTX-resistant Na currents of cultured DRG neurons display considerable differences in sensitivity to BAB (Van den Berg et al., 1995; Van den Berg et al., 1996). The inhibition of DRG Na currents is likely to contribute to the BAB anesthesia.

By comparison, the role of K channels in peripheral nerve anesthesia has not been extensively investigated. In large part, this reflects our rather sparse understanding of the K channels that are expressed in peripheral nerves and their role in the electrical excitability of these neurons. A variable combination of rapidly inactivating A-type (I_A) and slowly or non-inactivating (I_K) K currents are observed in most DRG neurons (Kostyuk et al., 1981; Gold et al., 1996; Akins and McCleskey, 1993). Pharmacological studies suggest that the I_A and I_K components of DRG K current can be further subdivided into several distinct components (Safronov et al., 1996). Current estimates suggest that as many as six different channels may contribute to the outward K current in these neurons (Gold et al., 1996). Dendrotoxin, a selective inhibitor of Kv1 channels (Harvey, 2001), induces repetitive action potential firing of sensory neurons by selectively inhibiting the delayed rectifier current (Hall et al., 1994; Penner et al., 1986; Stansfeld et al., 1986; Stansfeld et al., 1987; McAlexander and Undem, 2000; Glazebrook et al., 2002). The message encoding for Kv1.1 is present in the DRG (Beckh and Pongs, 1990; Glazebrook et al., 2002) and immunocytochemistry indicates that Kv1.1 channels are expressed in small DRG neurons (Hallows and Tempel, 1998; Ishikawa et al., 1999; Glazebrook et al., 2002). In addition, Kv1.1 knockout mice display hyperalgesia, consistent with an important role for these channels in nociception (Clark and Tempel, 1998).

In this study, we found that BAB produces a concentration-dependent inhibition of the whole-cell K current of cultured DRG neurons. Dendrotoxin K (DTX_K), a specific inhibitor of channels incorporating the Kv1.1 subunit (Robertson et al., 1996), inhibited the slowly inactivating K current of small DRG neurons indicating that Kv1.1 channels contribute to the delayed rectifier current in these cells. The mechanism of BAB inhibition was further investigated by examining its effect on Kv1.1 channels expressed in mammalian cells. BAB produces a concentration-dependent inhibition of the heterologously expressed Kv1.1 that is comparable to that observed for the native DRG K current. The data suggest that BAB inhibition of DRG Kv1.1 channels may contribute to the long-duration anesthesia produced by the epidural administration by this drug.

METHODS

Neonatal mice were sacrificed by decapitation in accordance with the standards of the Animal Ethical Committee of Leiden University Medical Center. The dorsal root ganglia (DRG) from all accessible levels of the spinal cord were collected and mechanically dissociated on a glass coverslip coated with poly-L-lysine (Mol. Wt. 70,000-150,000; Sigma) in 0.5 ml of F-12 Ham Kaighn's modified media supplemented with $CaCl_2$ (0.15 g/l), glutamine (0.29 g/l), $NaHCO_3$ (2.5 g/l), glucose (7.0 g/l) and 10% horse serum (GibcoBRL). The ganglia cells were allowed to attach to the coated glass coverslips for 2.5 h in a humidified 5% CO_2 atmosphere at 37°C after which an additional 2 ml of F-12 medium was added. The cells were cultured for 3-8 hours before selecting small (~20 μm) spherical neurons devoid of neurite outgrowth for patch clamp experiments.

The cDNAs of the rat[1] Kv1.1 potassium channel and the Kvβ1 subunit (Rettig et al., 1994), were subcloned into pcDNA3.1(-) (Invitrogen, San Diego, CA). The cDNA for eGFP (Clontech, Palo Alto, CA) was subcloned into pcDNA3.1(+) vectors (Invitrogen, San Diego, CA). tsA201 cells were cotransfected with cDNA encoding Kv1.1 and cDNA encoding for GFP, a green fluorescent marker that facilitates the identification of transfected cells, in a 1:1 ratio. The Kv1.1/GFP cDNA mixture was added to 0.5 ml of DMEM (Sigma) enriched with 10% fetal bovine serum (GibcoBRL) and 1% penicillin-streptomycin (Sigma). 25μl of 1,2-dioleoyl-sn-glycero-3-trimethylammonium-propane (DOTAP) (Roche Diagnistics GmbH, Mannheim, Germany) was slowly added and incubated for 15 min at room temperature. The cDNA/DOTAP mix was transferred to a 100-mm culture dish of 50% confluent tsA201 cells bathed in 10 ml of enriched DMEM. After 3 hours, the transfection solution was removed and replaced with 20 ml of enriched DMEM. After 24 hours, the cells were replated on glass coverslips. The cells were incubated an additional 12-24 hours before selecting GFP-positive cells (excitation: 488 nm, emission 507 nm) for use in patch-clamp studies. For experiments with the Kvβ1 subunit tsA201 cells were cotransfected with Kv1.1, GFP and Kvβ1 in a 1:1:2 ratio.

[1] In J Pharmacol Exp Ther (2003) 304:531-8 wrongly referred to as human

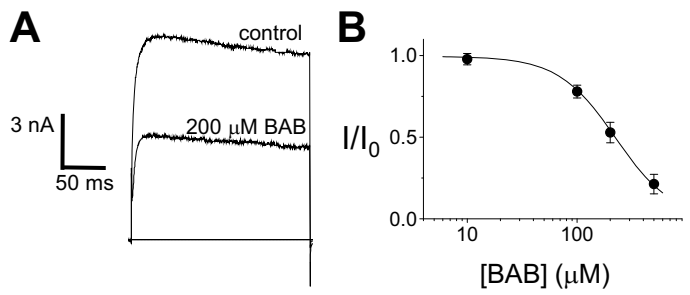

Figure 1. BAB inhibition of the native K current of small DRG neurons. A. Whole-cell patch clamp recording of K current from an acutely dissociated DRG neuron. Currents were elicited by depolarizing to +20 mV from a holding potential of –80 mV. Current recorded from the same cell is shown before and after the bath application of 200 μM BAB. B. Currents measured in the presence of BAB were normalized to drug-free controls and plotted versus the concentration. The smooth curve is a fit to the Hill equation $I/I_O = (1+([BAB]/IC_{50})^n)^{-1}$ with an IC_{50} of 223 ± 10 μM and Hill coefficient of 1.7 ± 0.1 (n = 32).

For the patch clamp experiments, a cover slip was mounted in a small perfusion chamber (75 μl) and continuously perfused (~300 μl/min) with extracellular solution. Patch pipettes were fabricated from borosilicate glass (Clark GC-150 TF-15) on a custom two-stage horizontal puller and had resistances between 1 and 2 MΩ. For DRG neurons the external solution consisted of (in mM): NaCl 35, KCl 5, $MgCl_2$ 3, HEPES 10, Sucrose 180, pH 7.35 (NaOH) with 300 nM tetrodotoxin (Sigma). The pipette solution was (in mM): NaCl 20, KCl 118, EGTA 5, HEPES 10, MgATP 2, pH 7.35 (NaOH). In experiments with tsA201 cells the extracellular solution consisted of (in mM): NaCl 136, KCl 2, $CaCl_2$ 1.5, $MgCl_2$ 1, HEPES 10, pH 7.4 (NaOH) . The pipette solution was (in mM): KCl 115, $MgCl_2$ 1, EGTA 10 HEPES 10, pH 7.4 (KOH). BAB was added to the extracellular solution from a stock of BAB in ethanol (1-500 μM). The final ethanol concentration in the extracellular solution was in all cases, including control experiments, 0.1 %. Dendrotoxin-K (Alomone, Jerusalem, Israel) was dissolved in distilled water before dilution in extracellular solution to a final concentration of 10 nM. Voltage pulses were generated by pClamp 8 (Axon Instruments, Foster City, CA) and recorded using a List EPC 7 patch-clamp amplifier (List Medical, Darmstadt, Germany). The series resistance of the patch pipettes was 75% compensated and current recordings were filtered at 3 kHz. All currents were leak subtracted using P/4 subtraction.

Membrane capacitance of the cells was estimated from the decay of the transient elicited by a 10 mV depolarizing voltage pulse from a –80 mV holding potential.

The concentration-inhibition data were fitted to 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. The activation data obtained from tail current measurements (Figure 4) were fitted to the Boltzmann equation: $I/I_o = (1+\exp-((V-V_{0.5})/k))^{-1}$ where V is the prepulse potential, $V_{0.5}$ the voltage at which the current is half maximally activated, and k is the slope factor. Unless otherwise stated the data are the Means $\pm$ SD for a given number (n) of cells.

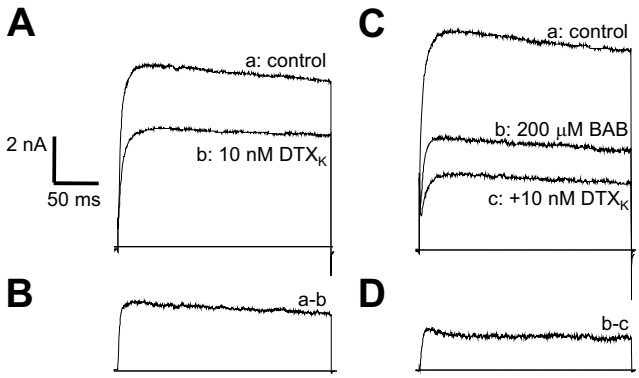

Figure 2. Inhibition of K current in a DRG neuron by dendrotoxin-K (DTX_K). A. K current elicited by depolarizing to +20 mV from a holding potential of –80 mV. Currents are shown before and after the bath application of 10 nM DTX_K. Currents were reduced 34 $\pm$ 7% by comparison to controls (n=7). B. The DTX_K-sensitive component of the DRG current (a-b) was isolated by subtracting the residual current remaining after application of DTX_K from the total DRG current in A. C. K current elicited by depolarizing to +20 mV from a holding potential of –80 mV. Currents are shown in control conditions, and after the sequential application of 200 μM BAB and 10 nM DTX_K + 200 μM BAB. D. The DTX_K-sensitive component as obtained by subtracting the current measured in the presence of BAB + DTX_K from the current measured in the presence of 200 μM BAB alone (b-c). Calibration bars in A apply to all panels.

RESULTS

BAB inhibition of the endogenous K current of dorsal root ganglion (DRG) neurons

To investigate the role of K channels in the BAB anesthesia, we used the patch-clamp technique to measure the whole-cell K current of small cultured DRG neurons (≈20 μm, 14 ± 3 pF, n=49), which are believed to represent the cell bodies of nociceptive pain fibers. The outward K currents were isolated by blocking sodium currents with tetrodotoxin (300 nM) and by applying test pulses close to the sodium reversal potential to minimize the contribution of the remaining TTX-resistant current. Calcium currents and calcium-activated currents were eliminated by removing external calcium and by including EGTA in the patch pipette. Cells were held at –80 mV and currents were elicited by depolarizing steps to +20 mV (Figure 1A). The majority of the K current in these cells appears to be best classified as the slowly inactivating or non-inactivating variety. Only a relatively minor contribution of the rapidly inactivating I_A component was observed in our study. Bath application of BAB (200 μM) reduced the amplitude of the current (Figure 1A). BAB inhibited the whole-cell K current of the small DRG neurons in a concentration-dependent fashion with an IC_{50} of 223 ± 10 μM (Figure 1B).

Kv1.1 channels contribute to the delayed rectifier current of DRG neurons

At least four distinct components have been shown to contribute to the slowly inactivating and sustained K current of DRG neurons but the molecular identities of the underlying channels have not been established (Safronov et al., 1996). Previous studies have shown that small DRG neurons express a slowly inactivating dendrotoxin-sensitive K current, suggesting that members of the Kv1 family may contribute to the delayed rectifier current in these cells (Hall et al., 1994; Penner et al., 1986; Stansfeld et al., 1986; Stansfeld et al., 1987; McAlexander and Undem, 2000; Glazebrook et al., 2002). To further investigate the channels underlying the slowly inactivating K current, we applied dendrotoxin-K (DTX_K), a specific inhibitor of Kv1.1 channels (Robertson et al., 1996). DTX_K (10 nM) decreased the whole-cell K current of DRG neurons by 34 ± 7 % (n=7) (Figure 2A). The DTX_K-sensitive component of the DRG current was

isolated by subtracting the current remaining after application of DTX_K from the total K current (Figure 2B). The DTX_K-sensitive component rapidly activated and displayed little inactivation during the 250 ms depolarization. The high sensitivity to DTX_K indicates that Kv1.1 channels, or heteromultimeric channels incorporating the Kv1.1 subunit, contribute to the slowly inactivating K current in these neurons.

We attempted to gain additional insight into the mechanism of BAB inhibition by investigating the overlap of the BAB- and DTX_K-sensitive components of the native DRG K current. In the absence of BAB, DTX_K (10 nM) inhibited 34% of the DRG current. This contrasts with what is observed in presence of 200 µM BAB (Figure 2C+2D) which significantly (p=0.001) reduced the relative amplitude of the DTX_K–sensitive current (20 $\pm$ 5 %, n=6). This suggests that the toxin and BAB inhibit a common component of the DRG K current. The relative amplitude of the DTX_K-sensitive current was further reduced by pre-applying 500 µM BAB (9 ± 4 %, n=6, $p=1\cdot10^{-5}$) providing additional support inhibition of DTX_K-sensitive current by BAB. The high selectivity of DTX_K indicates that the reduction in the amplitude of the native DRG K current, at least in part, results from the inhibition of Kv1.1 channels. In many cases, high concentrations of BAB (500 µM) completely inhibited the DRG K current suggesting that in addition to Kv1.1, other delayed rectifier currents were inhibited at these concentrations.

BAB inhibition of heterologously expressed Kv1.1 channels

To further investigate the mechanism of BAB inhibition, the cDNA encoding for Kv1.1 was heterologously expressed in tsA201 cells. At +20 mV, the Kv1.1 channels rapidly activated but only slowly inactivated similar to the DTX_K-sensitive component of DRG K current (Figure 3A). BAB inhibited the homomultimeric Kv1.1 channels in a concentration-dependent fashion with an IC_{50} of 238 $\pm$ 10 µM (Figure 3B), similar to what is observed for the native DRG current. In addition to reducing the amplitude, BAB caused the current to decay more rapidly. In the absence of drug, the current decay could be well fitted by a single exponential with a time constant of 373 $\pm$ 47 ms and a relative amplitude of 0.26 $\pm$ 0.02 (n = 4). This is likely to reflect the slow inactivation of Kv1.1 channels. After application of 200 µM BAB, the peak current was reduced by 53 $\pm$ 2% and the decay time course was found to be biexponential with time

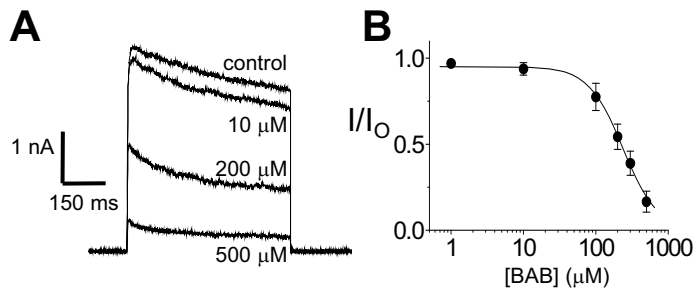

Figure 3. BAB inhibition of homotetrameric Kv1.1 channels. A. The Kv1.1 channel was heterologously expressed in tsA201 cells and currents were recorded by depolarizing to +20 mV from a holding potential of –80 mV. BAB (10 – 500 μM) progressively inhibits the whole-cell K current of the Kv1.1 channels. B. The current measured in the presence of BAB was normalized to drug-free control currents and plotted versus concentration. The smooth curve is a fit to the Hill equation with IC_{50} and Hill coefficient of 238 ± 10 μM and 1.8 ± 0.1 respectively. The data are means ± SD of 8-9 determinations at each concentration.

constants (relative amplitudes) of 36 ± 2 ms (0.11 ± 0.02) and 301 ± 56 ms (0.35 ± 0.01) respectively (n = 4). BAB induced a new rapid component of current decay and increased the relative amplitude of the slow component by comparison to drug-free controls. The data suggest that BAB may enhance the slow inactivation of Kv1.1. However, the onset of this component is too slow to account for the large reduction in the peak amplitude of the current. Other mechanisms, which have faster kinetics or that reduce the probability that a channel will open are likely to play a more prominent role in the BAB inhibition of these channels.

We also examined the effect of BAB on the reversal potential and activation gating of the Kv1.1 channels. For voltages between –80 and 0 mV the instantaneous current amplitudes were determined from the peak of the tail currents (Figure 4A) which were normalized and plotted versus the voltage (Figure 4B). Over this range of voltages, the current-voltage (I-V) relationship is linear with an extrapolated reversal potential of –89 ± 7 mV. Also plotted is the I-V relationship determined after the application of 200 μM BAB which has a reversal potential of -91 ± 11 mV. Although the peak current amplitudes are reduced, the reversal potentials are not significantly different indicating that BAB does not alter the selectivity of Kv1.1 channels (paired t-test, n=8).

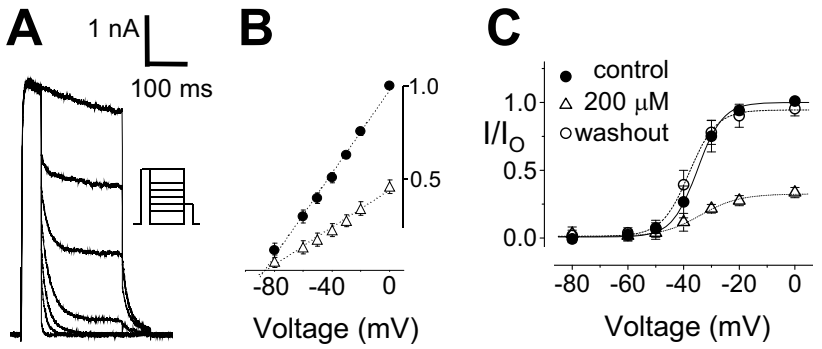

Figure 4. Effect of BAB on reversal potential and Kv1.1 activation. A. Currents were activated by stepping for 50 ms to 0 mV before applying a series of 200 ms test pulses to voltages between –80 and 0 mV followed by a step to –50 mV before returning to the holding potential. B. The current-voltage relationship was determined by measuring the peak amplitudes of the tails elicited by the variable voltage pulses. The currents were normalized to the current measured at 0 mV and plotted versus the voltage. The data are the means ± SD for 8 individual experiments. C. The activation was determined by plotting the normalized peak currents elicited by the –50 mV tail currents versus the prepulse voltage. The smooth curves are fits to a Boltzmann function with midpoints and slope factors of –35 ± 3 mV and 4.6 ± 1.0 mV for controls and –34 ± 3 mV and 7.0 ± 1.3 mV after application of 200 μM BAB (n = 8).

The effect of BAB on the activation of Kv1.1 was investigated by plotting the normalized peak amplitudes of the tail currents versus the prepulse potential (Figure 4C). In the absence of drug, the normalized current-voltage relationship was fitted to a Boltzmann function with a midpoint ($V_{0.5}$) and slope factor (k) of –35 ± 3 mV and 4.6 ± 1.0 mV respectively (n = 8). BAB (200 μM) reduced the tail current amplitudes but did not alter the midpoint of steady state activation ($V_{0.5}$ = -34 ± 3 mV). These effects were completely reversed upon removing BAB from the bath. The data indicate that in the presence of BAB, Kv1.1 channels display a reduced open probability or unitary conductance relative to the drug-free controls that cannot be attributed to a change in the voltage dependence of channel activation or selectivity. BAB may inhibit the Kv1.1 current through changes in the kinetics of gating or a reduction in the channel conductance.

BAB accelerates the activation and deactivation of Kv1.1 channels

Figure 5 shows Kv1.1 current measured at –30 mV before and immediately after the bath application of 200 μM BAB. The currents have been normalized to facilitate the comparison of the kinetics. BAB accelerates both the activation and deactivation time course of the current. To quantitatively compare the activation, we determined the time required for the current to reach its half-maximal amplitude. In eight paired experiments the half-maximal rise times were 10.7 $\pm$ 1.7 ms and 6.0 $\pm$ 1.0 ms before and after application of 200 μM BAB respectively. BAB significantly accelerates the rising phase of the current (paired t-test, $p<0.001$), an effect that cannot be attributed to a shift in the voltage dependence of activation (Figure 4B). We also examined the effect of BAB on the kinetics of activation at +20 mV, a voltage where the channels are maximally activated. At +20 mV the times to half of maximum amplitude were 2.6 $\pm$ 0.4 ms for controls and 2.4 $\pm$ 0.4 ms after application of BAB (n = 9). In the absence of drug, the half-maximal rise times at +20 mV were reduced by comparison to those measured at –30 mV and are consistent with the strong voltage dependence of Kv1.1 activation. Although the relative difference in the rise times of the control and drug-treated current at +20 mV is small, it was found to be significant in a paired t-test ($p<0.002$). This indicates that the more rapid rise

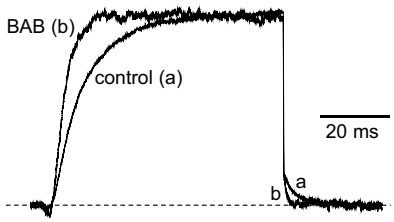

Figure 5. Superimposed Kv1.1 currents measured at –30 mV before and after application of 200 μM BAB. The amplitude of the current measured in the presence of BAB was normalized to the control in order to facilitate the comparison of the activation kinetics. The time course of activation was quantitatively evaluated by comparing the time required for the current to reach the half-maximal peak amplitude (see text).

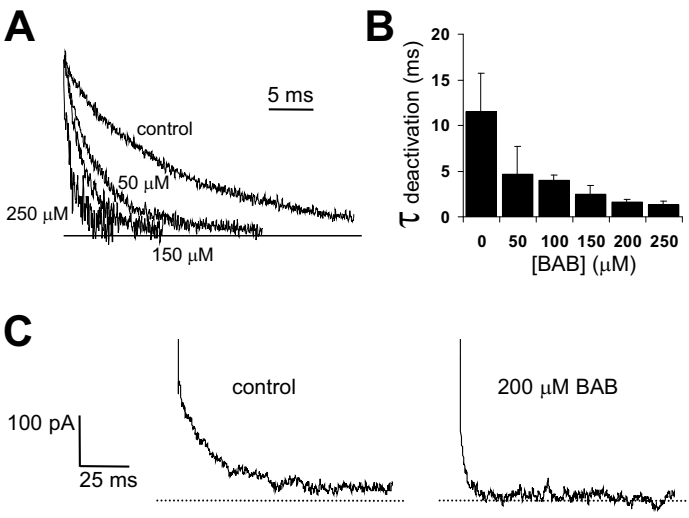

Figure 6. BAB accelerates the deactivation of Kv1.1 channels. A. Potassium currents evoked by a step to +20 mV for 50 ms and the tail currents measured upon return to –65 mV. The superimposed tail currents are shown before (control) and after the bath application of 50, 150 and 250 μM BAB. B. The decay of the tail current was fitted with a single exponential and the resulting time constants plotted versus the concentration (n = 7). C. Effect of BAB on the DTX_K-sensitive tail currents of cultured DRG neurons. The DTX_K-sensitive component was obtained by subtracting the current remaining after application of the toxin (10 nM) from the total K current (see Figure 2). The current of two cells are shown in the absence (left) and presence (right) of 200 μM BAB.

of the current observed after application of BAB results from a genuine increase in the activation kinetics and does not reflect contamination by deactivation, which is likely to contribute to the apparent activation kinetics at the less depolarized (-30 mV) test potential.

In addition to its effects on activation, BAB also enhances the deactivation of Kv1.1. Figure 6A shows a family of normalized Kv1.1 tail currents measured before and after application of BAB (50 – 250 μM). BAB accelerates the deactivation of the channels in a concentration-dependent fashion (Figure 6B). Also shown are two typical tail currents of DRG neurons in the absence and presence of 200 μM BAB (Figures 6C). The tail currents are well fitted by a single exponential with time constants (τ) of 17.0 ± 5.9 ms (n = 8) in the absence and

4.0 ± 1.4 ms (n = 9) in the presence of BAB. BAB produces a similar increase in the deactivation of both the heterologously expressed Kv1.1 and the native DRG K current. Overall, the data indicate that changes in both activation and deactivation kinetics may contribute to the BAB inhibition of Kv1.1 channels.

Effect of the Kvβ subunit on the gating and BAB sensitivity of Kv1.1

Previous studies have shown that co-expressing Kv1.1 and Kvβ subunits result in a rapidly inactivating A-type current. The N-terminus of the Kvβ1 subunit is proposed to act as an inactivation particle that occludes the internal vestibule of activated Kv1.1 channels (Rettig et al., 1994). We were therefore interested in determining the effects of the Kvβ1 subunit and rapid inactivation on the BAB sensitivity of Kv1.1 channels. Co-expressing the Kvβ1 and Kv1.1 subunits resulted in current that rapidly inactivated similar to what has been previously reported for this oligomeric channel (Figure 7A). Similar to the Kv1.1 channels, BAB inhibited the Kv1.1/Kvβ1 channel in a concentration-dependent inhibition fashion. The peak currents measured before and after application of BAB were normalized to drug-free controls and plotted versus the BAB concentration (Figure 7B). BAB inhibited the current with an IC_{50} and Hill coefficient of 343 $\pm$ 10 μM and 2.1 $\pm$ 0.2, respectively (n = 17). The BAB sensitivity of Kv1.1 (IC_{50} = 238 μM) was significantly reduced by co-expressing the channel with the Kvβ1 subunit. It is not clear if the reduced inhibition results from a conformational change in Kv1.1 induced by the Kvβ1 subunit or if rapid inactivation somehow weakens BAB binding.

To further investigate the role of inactivation in the BAB inhibition we examined its effects on the steady-state inactivation of the Kv1.1/Kvβ1 channel. Depolarizing prepulses were used to inactivate the channels before applying a standard test pulse to assay availability (Figure 7C, inset). The currents elicited by the test pulses were normalized to controls measured after prolonged hyperpolarization to –80 mV and plotted versus the prepulse voltage. The relative amplitudes of the test currents progressively decrease with prepulse voltage consistent with an increase in steady-state inactivation. The smooth curves are fits to the Boltzmann function with a midpoint ($V_{0.5}$) and slope factor (k) of –53 $\pm$ 3 mV and 3.4 $\pm$ 0.2 mV respectively (n = 4). BAB (200 μM) reduces the maximal current amplitude measured at hyperpolarized voltages by 12% but

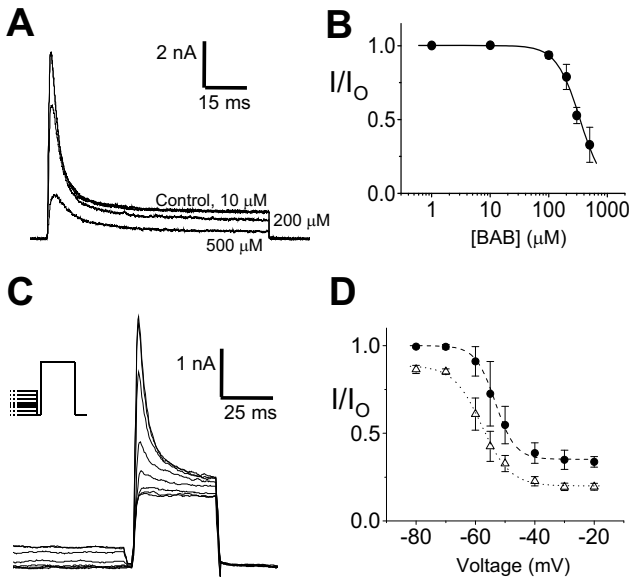

Figure 7. BAB effects on Kv1.1 channels co-expressed with the Kvβ1 subunit. A. Whole-cell current of cells expressing the Kv1.1 α and Kvβ1 subunits. Currents were elicited by depolarizing to +20 mV from a holding potential of –80 mV. Currents are shown before (control) and after bath application of 10, 200 and 500 μM BAB. B. The peak amplitude of the currents measured in the presence of BAB were normalized to the drug-free controls and plotted versus [BAB]. The smooth curve is a fit to the Hill equation with an IC_{50} of 343 ± 10 μM and coefficient of 2.1 ± 0.2. The data are means ± SD of 7 or 8 determinations at each concentration. C. The steady state inactivation was measured by applying 500 ms prepulses to voltages between –80 and –20 mV. Only the last 30 ms of the prepulses are shown for clarity. A short hyperpolarization to –80 mV for 4 ms was used to fully deactivate the channels before applying a standard test pulse to +50 mV. The peak amplitudes of the test currents were normalized to controls measured directly from the –80 mV holding voltage and plotted versus the prepulse potential. The smooth curves are fits to the Boltzmann function with midpoints and slope factors of –53 ± 3 mV and 3.4 ± 0.2 mV for controls (filled circles) and –56 ± 2 mV and 4.8 ± 0.7 mV after applying 200 μM BAB (open triangles) (n=4).

does not significantly alter the midpoint ($V_{0.5}$ = -56 ± 2 mV) or voltage sensitivity (k = 4.8 ± 0.7 mV) of inactivation. Hyperpolarizing shifts in steady-state inactivation are typical of drugs that preferentially affect channel inactivation. BAB does not inhibit the Kv1.1/Kvβ1 by preferentially interacting with the

inactivated state of the channel. Furthermore, the BAB inhibition persists at hyperpolarized voltages (-80 mV) where few of the Kv1.1/Kvβ1 channels are predicted to be inactivated. Overall, the data suggest that rapid inactivation does not play a prominent role in the BAB inhibition of Kv1.1. Conformational changes in the Kv1.1 channel induced by interaction with Kvβ1 may account for the reduced BAB sensitivity observed in these studies.

DISCUSSION

In this study, we investigated the anesthetic sensitivity of the slowly inactivating K current of small cultured DRG neurons of neonatal mice. The majority of the whole-cell K current of these neurons rapidly activates and slowly inactivates and has properties that are consistent with a delayed rectifier type current. We further investigated the role of Kv1 channels using DTX_K, a specific inhibitor of channels incorporating the Kv1.1 subunit (Wang et al., 1999). DTX_K inhibited 34% of the slowly inactivating DRG K current consistent with an important contribution of Kv1.1 to the delayed rectifier current in these neurons. This finding is in agreement with a recent study showing that DTX_K inhibits the delayed rectifier current of C-type neurons (Glazebrook et al., 2002). BAB inhibited the slowly inactivating K current of DRG neurons in a dose-dependent fashion with an IC_{50} of 223 μM. Our data indicate substantial overlap in the inhibition produced by BAB and DTX_K, supporting the conclusion that Kv1.1 channels contribute to the BAB-sensitive current in these small DRG neurons. The inhibition of Kv1.1 channels occurs within the range of BAB concentrations realized in the epidural space during the clinical administration of this drug (Grouls et al., 1997).

BAB inhibition of Kv1.1 channels

To better understand the mechanism we examined the effects of BAB on the current of heterologously expressed Kv1.1 channels. Kv1.1 rapidly activates and slowly inactivates similar to what is observed for the DTX_K-sensitive current of

DRG neurons. BAB inhibited Kv1.1 with an IC_{50} of 238 μM, which is nearly identical to what was observed for the inhibition of the native DRG K current (IC_{50} = 223 μM). In addition to reducing the current amplitude, BAB accelerated the activation and deactivation kinetics of Kv1.1 but did not produce any change in the midpoint of activation. A similar BAB-induced increase in the kinetics of deactivation was observed for the native DRG K current. Assuming a simple two state model for activation gating suggests that the opening and closing kinetics are equally enhanced by BAB. Such symmetrical changes in opening/closing rates are difficult to explain by the preferential binding of BAB to either the closed or open conformations of the channel. BAB does not appear to act by a state-dependent binding mechanism.

Several mechanisms could potentially explain the BAB inhibition of Kv1.1 channels. We initially considered that the inhibition produced by BAB could result from a channel blocking mechanism. However, simple blocking models generally predict slower deactivation because the channels often cannot close until the drug dissociates from its binding site (Armstrong, 1971). This is clearly inconsistent with the observed effects of BAB on either the heterologously expressed Kv1.1 or native DRG tail currents which were faster in the presence of the drug. BAB also induced a slow decay in the sustained current of heterologously expressed Kv1.1 that may be linked to the slow inactivation of these channels. However, the time course of this decay (τ = 36 ms) is too slow to account for the reduction in the amplitude of the peak current observed after the application of BAB. The observed kinetic changes also indicate that a reduction of single channel conductance cannot be the sole mechanism. Rather the data appears to favor an allosteric mechanism in which BAB biases the channels towards the closed state. Rapid deactivation may effectively stabilize the channels in closed (non-conducting) conformations and could account for the BAB-induced reduction in the amplitude of Kv1.1 and native K current in DRG neurons.

Co-expression of the Kvβ1 subunits confers rapid N-type inactivation on the slowly inactivating Kv1.1 channels (Rettig et al., 1994; Heinemann et al., 1996) and the message encoding for several of the Kvβ subunits is present in the sensory neurons of nodose ganglion (Glazebrook et al., 2002). Consistent with these previous findings we found that co-expressing the Kvβ1 subunit resulted in

rapid but incomplete inactivation of Kv1.1. This rapid inactivation contrasts with the native DTX_K-sensitive component of DRG K current, which slowly inactivates similar to what is observed when Kv1.1 channels are expressed alone. Our data therefore suggest that the endogenous Kv1.1 channels expressed in DRG neurons may not associate with the Kvβ1 subunit. Alternatively, Kv1.1 subunits may form heteromultimers with other Kv1 subunits (Isacoff et al., 1990; Ruppersberg et al., 1990) resulting in channels that retain sensitivity to DTX_K (Wang et al., 1999) but that are not strongly regulated by the Kvβ subunit. The rapidly inactivating Kv1.1/Kvβ1 oligomeric channel (IC_{50} = 343 μM) is considerable less sensitive to BAB than Kv1.1 (IC_{50} = 238 μM). BAB does not alter the kinetics of the current decay or steady state inactivation of the Kv1.1/Kvβ1 channels suggesting that N-type inactivation is not tightly linked to the BAB inhibition. Rather the data suggest that interaction with the Kvβ1 subunit may induce a conformational change in Kv1.1 that weakens BAB binding or that indirectly modulates the inhibitory mechanism.

Role of Kv1.1 channels in the long duration BAB anesthesia of DRG neurons

Voltage-gated K currents play an integral role in setting the resting membrane potential and in action potential repolarization, and are important determinates of spike frequency and burst adaptation (Rudy, 1988). Small DRG neurons, which are believed to reflect the cell bodies of unmyelinated C-fibers, display Kv1.1 immunoreactivity and the DRG contains RNA encoding for Kv1.1 channels (Beckh and Pongs, 1990; Hallows and Tempel, 1998;Ishikawa et al., 1999). The importance of Kv1 channels to the electrical excitability of DRG neurons is illustrated by studies showing that DTX_α, an inhibitor of several of the Kv1 channels, induces rapid repetitive firing of sensory neurons (McAlexander and Undem, 2000; Stansfeld et al., 1986; Glazebrook et al., 2002). This is further supported by studies of Kv1.1 null mice, which display hyperalgesia and reduced sensitivity to opiate therapy, symptoms frequently associated with neuropathic pain (Clark and Tempel, 1998). It suggests that the absence of Kv1.1 in the null mice causes sensory neurons to become hyperexcitable, similar to what is observed after application of DTX. This is consistent with data showing that the delayed rectifier current makes an important contribution to the resting membrane potential of small DRG neurons (Safronov et al., 1996). Overall, these

previous studies appear to be in good agreement of our data indicating that Kv1.1 channels contribute to the delayed rectifier current of DRG neurons.

A possibility is that like DTX, BAB inhibition of Kv1.1 may paradoxically increase rather than suppress the electrical excitability of DRG neurons. This might be expected to cause hyperalgesia similar to what was observed in the Kv1.1 null mice (Clark and Tempel, 1998). However, other effects of BAB should also be taken into account. Previous studies indicate that in addition to K channels, the endogenous Na currents of DRG neurons are also sensitive to BAB. At least two Na channels are known to contribute to the electrical excitability of small DRG neurons. $Na_V1.7$ is a rapidly gating TTX-sensitive Na channel and $Na_V1.8$ is a slowly gating TTX-resistant Na channel (Waxman et al., 1999). Although both channels are generally believed to contribute to the Na current of sensory neurons, Nav1.8 appears to be exclusively expressed in the cell bodies of C-fibers (Akopian et al., 1996; Sangameswaran et al., 1996). Recent work has demonstrated that low-frequency repetitive stimulation (1-2 Hz) significantly reduces the steady state availability of the $Na_V1.8$ channels, an effect that appears to be due to the unusually rapid onset of slow inactivation in these channels (Vijayaragavan et al., 2001). By comparison, $Na_V1.7$ channels are considerably less sensitive to repetitive stimulation and are more resistant to slow inactivation. Similar observations have been made for the native TTX-sensitive and TTX-resistant Na currents of DRG neurons (Rush et al., 1998;Scholz et al., 1998). BAB causes a hyperpolarizing shift of the steady-state inactivation of the TTX-sensitive Na currents (Van den Berg et al., 1995;Van den Berg et al., 1996). This is predicted to reduce the availability of these Na channels, an affect that would be exacerbated by the inhibition of Kv1.1 and depolarization of the resting membrane potential. Because of the substantial differences in the voltage dependence of the $Na_V1.7$ and $Na_V1.8$ channels, even a slight depolarization of the resting membrane potential would tend to selectively inactivate $Na_V1.7$ and therefore increase the relative amplitude of the slower gating TTX-resistant currents. This could have important implications for the firing behavior of DRG neurons (cf. Vijayaragavan et al., 2001). Inhibition of Kv1.1 may also delay and weaken the repolarization of DRG neurons following an action potential similar to what has been previously observed with DTX_K (Glazebrook et al., 2002). Delayed repolarization would tend to slow the

recovery of inactivated Na channels and further increase the refractory period for action potential firing.

Our current working hypothesis is that BAB influences the availability of ion channels responsible for maintaining the high electrical excitability of DRG neurons. BAB inhibition of Kv1.1 and peripheral nerve Na channels may contribute to the long duration anesthesia associated with the epidural administration of this drug.

ACKNOWLEDGMENTS

We thank Prof. Thomas Schmidt (Leiden University, The Netherlands) for providing us the GFP-cDNA and Dr. Manuel Covarrubias (Jefferson Medical College, Philadelphia, PA) for providing us the cDNAs for Kv1.1 and Kvβ1 and for commenting the manuscript.

References

Akins PT and McCleskey EW (1993) Characterization of potassium currents in adult rat sensory neurons and modulation by opioids and cyclic AMP. Neuroscience **56**:759-769.

Akopian AN, Sivilotti L, and Wood JN (1996) A tetrodotoxin-resistant voltage-gated sodium channel expressed by sensory neurons. Nature **379**:257-262.

Armstrong CM (1971) Interaction of tetraethylammonium ion derivatives with the potassium channel of giant axon. J Gen Physiol **58**:413-437.

Beckh S and Pongs O (1990) Members of the RCK potassium channel family are differentially expressed in the rat nervous system. EMBO J. **9**:777-782.

Clark JD and Tempel BL (1998) Hyperalgesia in mice lacking the Kv1.1 potassium channel gene. Neurosci.Lett. **251**:121-124.

Glazebrook PA, Ramirez AN, Schild JH, Shieh CC, Doan T, Wible BA, Kunze DL (2002) Potassium channels Kv1.1, Kv1.2 and Kv1.6 influence excitability of rat visceral sensory neurons. J. Physiol (Lond) **541**: 467-482.

Gold MS, Shuster MJ, and Levine JD (1996) Characterization of six voltage-gated K+ currents in adult rat sensory neurons. J.Neurophysiol. **75**:2629-2646.

Grouls R, Korsten E, Ackerman E, Hellebrekers L, van Zundert, Breimer D. (2000) Diffusion of n-butyl-p-aminobenzoate (BAB) and bupivacaine through the human dura-arachnoid mater in vitro. Eur. J. Pharm. Sci. 12:125-131.

Grouls RJ, Meert TF, Korsten HH, Hellebrekers LJ, Breimer DD (1997) Epidural and intrathecal n-butyl-p-aminobenzoate solution in the rat. Comparison with bupivacaine. Anesthesiology **86**:181-187.

Grupe A, Schroter KH, Ruppersberg JP, Stocker M, Drewes T, Beckh S, and Pongs O (1990) Cloning and expression of a human voltage-gated potassium channel. A novel member of the RCK potassium channel family. EMBO J. **9**:1749-1756.

Hall A, Stow J, Sorensen R, Dolly JO, and Owen D (1994) Blockade by dendrotoxin homologues of voltage-dependent K+ currents in cultured sensory neurones from neonatal rats. Br.J.Pharmacol. **113**:959-967.

Hallows JL and Tempel BL (1998) Expression of Kv1.1, a Shaker-like potassium channel, is temporally regulated in embryonic neurons and glia. J.Neurosci. **18**:5682-5691.

Harper AA and Lawson SN (1985) Conduction velocity is related to morphological cell type in rat dorsal root ganglion neurones. J.Physiol (Lond) **359**:31-46.

Harvey AL (2001) Twenty years of dendrotoxins. Toxicon 2001.Jan.;39.(1):15.-26. **39**:15-26.

Heinemann SH, Rettig J, Graack HR, and Pongs O (1996) Functional characterization of Kv channel beta-subunits from rat brain. J.Physiol **493 (Pt 3)**:625-633.

Isacoff EY, Jan YN, and Jan LY (1990) Evidence for the formation of heteromultimeric potassium channels in Xenopus oocytes. Nature **345**:530-534.

Ishikawa K, Tanaka M, Black JA, and Waxman SG (1999) Changes in expression of voltage-gated potassium channels in dorsal root ganglion neurons following axotomy. Muscle Nerve **22**:502-507.

Korsten HH, Ackerman EW, Grouls RJ, van Zundert AA, Boon WF, Bal F, Crommelin MA, Ribot JG, Hoefsloot F, and Slooff JL (1991) Long-lasting epidural sensory blockade by n-butyl-p-aminobenzoate in the terminally ill intractable cancer pain patient. Anesthesiology **75**:950-960.

Kostyuk PG, Veselovsky NS, Fedulova SA, and Tsyndrenko AY (1981) Ionic currents in the somatic membrane of rat dorsal root ganglion neurons-III. Potassium currents. Neuroscience **6**:2439-2444.

Kuroda Y, Nasu H, Fujiwara Y, Nakagawa T (2000) Orientations and locations of local anesthetics benzocaine and butamben in phospholipid membranes as studied by 2H NMR spectroscopy. J. Membr. Biol. 177:117-28.

McAlexander MA and Undem BJ (2000) Potassium channel blockade induces action potential generation in guinea-pig airway vagal afferent neurones. J.Auton.Nerv.Syst. **78**:158-164.

McCarthy RJ, Kerns JM, Nath HA, Shulman M, Ivankovich AD (2002) The antinociceptive and histologic effect of sciatic nerve blocks with 5% butamben suspension in rats. Anesth. Analg. 94:711-716.

Penner R, Petersen M, Pierau FK, and Dreyer F (1986) Dendrotoxin: a selective blocker of a non-inactivating potassium current in guinea-pig dorsal root ganglion neurones. Pflugers Arch. **407**:365-369.

Rettig J, Heinemann SH, Wunder F, Lorra C, Parcej DN, Dolly JO, and Pongs O (1994) Inactivation properties of voltage-gated K+ channels altered by presence of beta-subunit. Nature **369**:289-294.

Robertson B, Owen D, Stow J, Butler C, and Newland C (1996) Novel effects of dendrotoxin homologues on subtypes of mammalian Kv1 potassium channels expressed in Xenopus oocytes. FEBS Lett. **383**:26-30.

Roy ML and Narahashi T (1992) Differential properties of tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels in rat dorsal root ganglion neurons. J.Neurosci. **12**:2104-2111.

Rudy B, (1988) Diversity and ubiquity of K channels. Neuroscience **25**:729-49.

Ruppersberg JP, Schroter KH, Sakmann B, Stocker M, Sewing S, and Pongs O (1990) Heteromultimeric channels formed by rat brain potassium-channel proteins. Nature **345**:535-537.

Rush AM, Brau ME, Elliott AA, and Elliott JR (1998) Electrophysiological properties of sodium current subtypes in small cells from adult rat dorsal root ganglia. J.Physiol (Lond) **511**:771-789.

Safronov BV, Bischoff U, and Vogel W (1996) Single voltage-gated K+ channels and their functions in small dorsal root ganglion neurones of rat. J.Physiol **493**:393-408.

Sangameswaran L, Delgado SG, Fish LM, Koch BD, Jakeman LB, Stewart GR, Sze P, Hunter JC, Eglen RM, and Herman RC (1996) Structure and function of a novel voltage-gated, tetrodotoxin-resistant sodium channel specific to sensory neurons. J.Biol.Chem. **271**:5953-5956.

Scholz A, Kuboyama N, Hempelmann G, and Vogel W (1998) Complex blockade of TTX-resistant Na+ currents by lidocaine and bupivacaine reduce firing frequency in DRG neurons. J.Neurophysiol. **79**:1746-1754.

Shulman M, Lubenow TR, Nath HA, Blazek W, McCarthy RJ, and Ivankovich AD (1998) Nerve blocks with 5% butamben suspension for the treatment of chronic pain syndromes. Reg Anesth.Pain Med. **23**:395-401.

Stansfeld CE, Marsh SJ, Halliwell JV, and Brown DA (1986) 4-Aminopyridine and dendrotoxin induce repetitive firing in rat visceral sensory neurones by blocking a slowly inactivating outward current. Neurosci.Lett. **64**:299-304.

Stansfeld CE, Marsh SJ, Parcej DN, Dolly JO, and Brown DA (1987) Mast cell degranulating peptide and dendrotoxin selectively inhibit a fast-activating potassium current and bind to common neuronal proteins. Neuroscience **23**:893-902.

Van den Berg RJ, Van Soest PF, Wang Z, Grouls RJ, and Korsten HH (1995) The local anesthetic n-butyl-p-aminobenzoate selectively affects inactivation of fast sodium currents in cultured rat sensory neurons. Anesthesiology **82**:1463-1473.

Van den Berg RJ, Wang Z, Grouls RJ, and Korsten HH (1996) The local anesthetic, n-butyl-p-aminobenzoate, reduces rat sensory neuron excitability by differential actions on fast and slow Na+ current components. Eur.J.Pharmacol. **316**:87-95.

Vijayaragavan K, O'Leary ME, and Chahine M (2001) Gating properties of Na(v)1.7 and Na(v)1.8 peripheral nerve sodium channels. J.Neurosci. **21**:7909-7918.

Wang FC, Bell N, Reid P, Smith LA, McIntosh P, Robertson B, and Dolly JO (1999) Identification of residues in dendrotoxin K responsible for its discrimination between neuronal K+ channels containing Kv1.1 and 1.2 alpha subunits. Eur.J.Biochem. **263**:222-229.

Waxman SG, Dib-Hajj S, Cummins TR, Black JA. (1999) Sodium channels and pain. Proc. Natl. Acad. Sci. 96:7635-9