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

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Citation

Beekwilder, J. P. (2008, May 22). *'Butamben, a specific local anesthetic and aspecific ion channel modulator'*. Retrieved from <https://hdl.handle.net/1887/12865>

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CHAPTER 4

THE LOCAL ANESTHETIC BUTAMBEN INHIBITS AND ACCELERATES LOW-VOLTAGE ACTIVATED T-TYPE CURRENTS IN SMALL SENSORY NEURONS.

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Anesth Analg (2006) 102:141-5

ABSTRACT

Butamben (BAB) is a local anesthetic, which can be used in epidural suspensions for long-term selective suppression of dorsal root pain signal transmission and in ointments for the treatment of skin pain. Previously, high-voltage activated (HVA) N-type calcium channel inhibition has been implicated in the analgesic effect of BAB. In the present study we show that low-voltage activated (LVA) or T-type calcium channels may also contribute to this effect. Typical transient T-type barium currents, selectively evoked by low-voltage (-40 mV) clamp stimulation of small (~20 μm diameter) dorsal root ganglion neurons from newborn mice, were inhibited by BAB with an IC_{50} value of ~200 μM . Furthermore, 200 μM BAB accelerated T-type current activation, deactivation and inactivation kinetics, comparable to earlier observations for N-type calcium channels. Finally, 200 μM BAB had no effect on the midpoint potential and slope factor of the activation curve, while it caused a ~3 mV hyperpolarizing shift of the inactivation curve, without affecting the slope factor. We conclude that BAB inhibits T-type calcium channels with a mechanism associated with channel kinetics acceleration.

INTRODUCTION

Epidural suspensions of the hydrophobic local anesthetic n-butyl-p-aminobenzoate (BAB), also known as butamben, have been shown to selectively inhibit dorsal root pain signal transmission for periods of months (Korsten et al., 1991), while butamben ointments are used for topical treatment of skin pain and itching. In a previous study of BAB's action mechanism we found that BAB inhibited high-voltage activated (HVA) calcium channels in dorsal root ganglion (DRG) neurons from newborn mice (Beekwilder et al., 2005). We studied both the effects of BAB on the calcium and barium current, which consists mainly of HVA N-type current. In the present study we have extended our investigations of BAB effects on calcium channels to the effects of BAB on low-voltage activated (LVA) or T-type calcium channels of mouse DRG neurons.

Although the T-type calcium currents have already been discovered in sensory neurons in the early eighties of last century and particularly in the small-size pain-sensing neurons (Carbone and Lux, 1984), their exact role has never been made clear. In general, T-type currents are believed to be involved in neuronal pacemaker activity and in promoting calcium entry during short action potentials; for a review see Yunker and McEnery (2003). However, in recent studies T-type calcium currents have been shown to play a role in pain signalling and have also been recognized as therapeutic targets (Bilici et al., 2001; Todorovic et al., 2001; Todorovic et al., 2004; Bourinet et al., 2005). Therefore, it is of interest to investigate whether T-type calcium channels are inhibited by BAB.

The distinct biophysical properties of T-type calcium channels (low voltage activation and its transient nature due to fast inactivation) make it possible to separate effects of BAB on T-type channels from non-T-type calcium channels without the use of ion channel blockers. We were also interested in the question whether an inhibition of T-type channels would be accompanied by an acceleration of channel kinetics, as has been observed in our earlier studies for various channel types, including native and cloned Kv1.1 channels (Beekwilder et al., 2003) and native N-type calcium channels (Beekwilder et al., 2005). The patch-clamp technique in the whole-cell voltage-clamp configuration allowed us

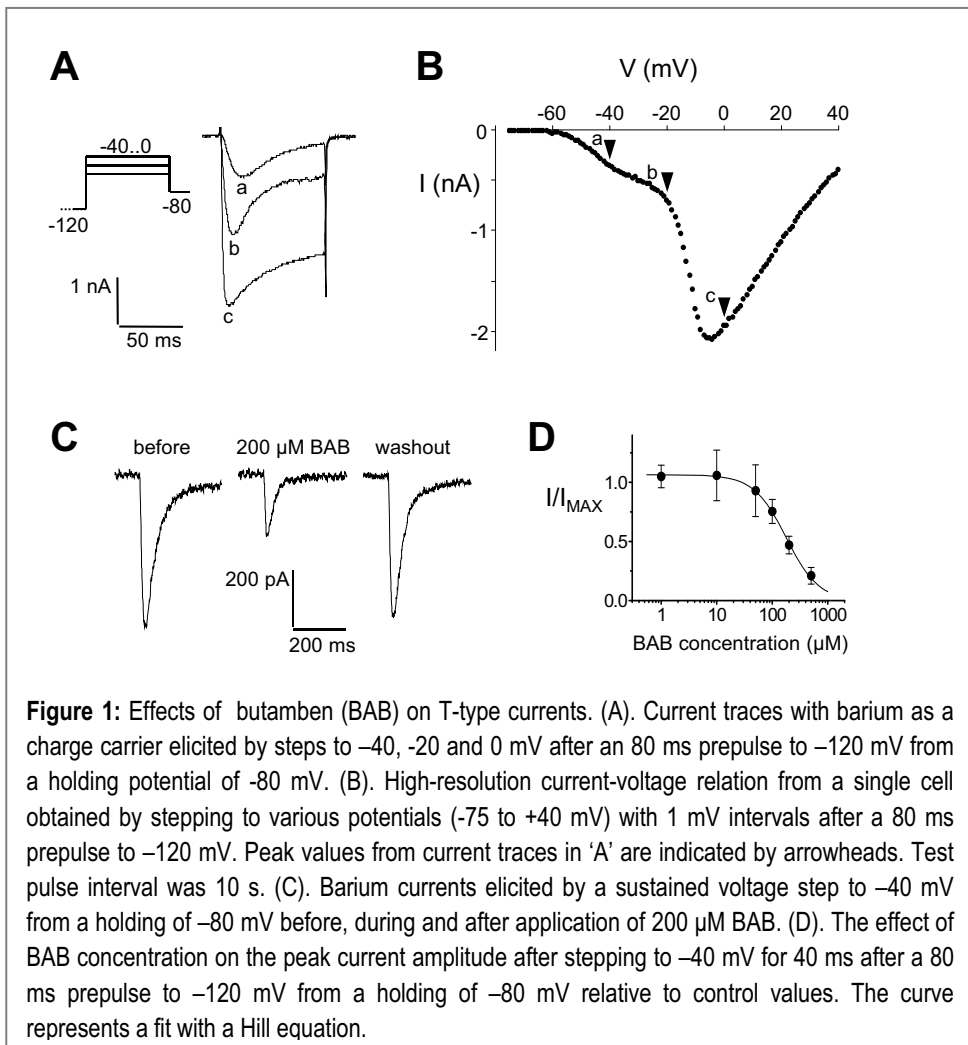
to establish that BAB indeed inhibits T-type currents and, at the same time, accelerates T-type current kinetics in small-size (~20 μm diameter) dorsal root ganglion neurons from neonatal mice. Functional implications of these findings are discussed.

METHODS

Cell culture, electrophysiology and data analysis were as described in detail elsewhere (Beekwilder et al., 2005). In short, small spherical neurons (~20 μm in diameter), mainly comprising nociceptive neurons (Todorovic et al., 2001), dissociated from dorsal root ganglia (DRG) of neonatal mice were patch-clamped within 8 h of culture. Patch pipettes of borosilicate glass had resistances of 2.0 to 2.5 M Ω . Giga-seals were made in a microbath of ~75 μl , continuously perfused (~300 $\mu\text{l}/\text{min}$) with standard extracellular solution containing (in mM): NaCl 145, KCl 5, CaCl₂ 2, MgCl₂ 1, HEPES 10, pH 7.4 (NaOH). The pipette solution contained (in mM): Cs-methanesulfonate 103, MgCl₂ 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 calcium channel currents were measured as barium currents during extracellular perfusion with (in mM): TEA-Cl 160, HEPES 10, EGTA 0.1, BaCl₂ 5, pH 7.4 (TEA-OH).

To minimize offset caused by the low Cl⁻ pipette solution, the pipette holder (Buisman et al., 1990) contained a Cl⁻ rich solution at the Ag/AgCl electrode. Experiments were carried out at room temperature (~23°C) with a List EPC 7 amplifier (3 kHz filtering) and controlled by pClamp software (Axon Instruments, Foster City, CA). The membrane capacitance of the selected DRG neurons was ~14 pF, the series resistance was largely (80-90%) compensated and the records were leak subtracted.

Butamben (BAB, OPG Farma, Utrecht, The Netherlands) was added to the extracellular solution from a stock of BAB in ethanol (1-500 mM). Normalized data were corrected for rundown in the presence of vehicle (0.1 % alcohol) at all



potentials measured in control experiments ($n=8$). At test pulses of -40 mV rundown was $< 3\%$ in 12 min.

The Hill equation, $I/I_o = (1 + ([\text{BAB}]/IC_{50})^n)^{-1}$, was fitted to the concentration-inhibition data, where IC_{50} is the concentration at which the current is reduced by 50% and n is the Hill coefficient. The Boltzmann equation, $I/I_o = (1 + \exp((V - V_{0.5})/k))^{-1}$, with V the prepulse potential, $V_{0.5}$ the midpoint potential at which the current is half maximal, and k the slope factor, was fitted to the steady-state inactivation data. T-type current kinetics were fitted with a m^2h Hodgkin-Huxley type model: $I(t) = (m^*)^2 \cdot (1 - \exp(-t/\tau_m))^2 \cdot (\exp(-t/\tau_h) + h^* \cdot (1 - \exp(-t/\tau_h))) \cdot A$, where m^* is partial steady-state activation at -40 mV,

obtained from the experiment in Fig. 2C,D, h^* is a free parameter representing the partial steady-state inactivation and A is an amplitude factor. The time constants for the m - and h -gate are τ_m and τ_h , respectively.

Results are presented as Means \pm Standard Deviations for n cells (unless mentioned otherwise) and compared using paired or independent t -tests with the level of significance $P = 0.05$.

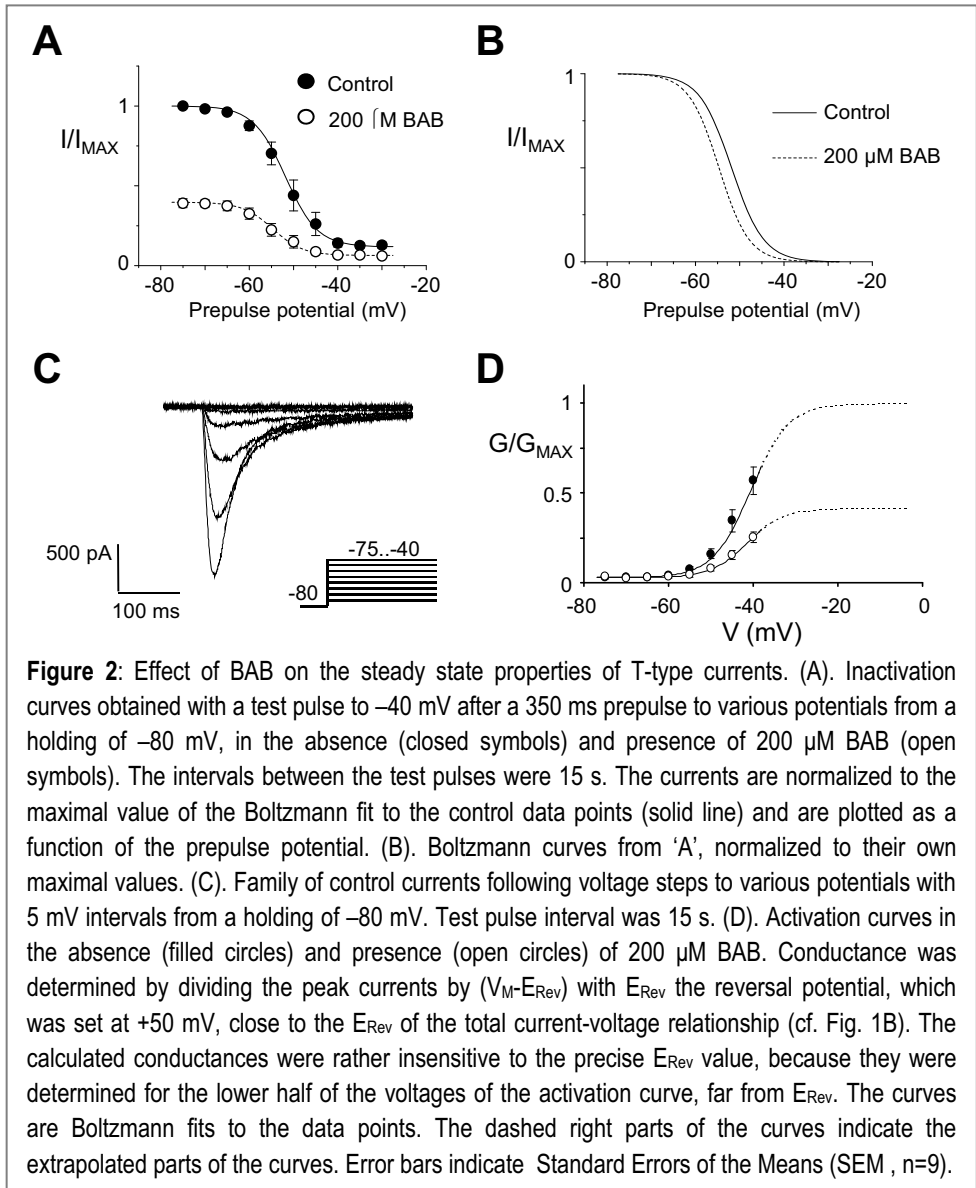
RESULTS

BAB inhibits T-type calcium channels

DRG neurons express both low-voltage (LVA) and high-voltage activated (HVA) calcium channels. LVA (T-type) channels activate around -55 mV, whereas HVA channels activate at more positive potentials (>-30 mV). Figure 1A shows these properties. Barium currents were evoked by voltage steps to various potentials from a holding potential of -80 mV, whereby the test pulses were preceded by a prepulse of -120 mV. Both the rise and decay of the current were strongly voltage dependent. Plotting the peak values of the currents against the various potentials (-75 ... $+40$ mV) with 1 mV intervals results in a high-resolution current-voltage relation of the total current (Fig. 1B). The distinct activation voltage ranges of the LVA and HVA currents can be recognized from the two-component character of the figure. This property allowed us to discriminate effects of BAB on LVA and HVA currents.

The LVA barium current at membrane voltages of -40 mV is carried mainly through T-type calcium channels and is characterized by a relatively fast and nearly-complete inactivation. Other indications confirming that the current we observed was T-type include the crossing of the current traces at successive voltage steps (cf. Fig. 2C) and a relatively slow deactivation compared to currents elicited by stronger depolarizing steps to 0 mV (cf. Fig. 3C) (Randall and Tsien,

1997). The amplitude of the peak barium current, elicited by voltage pulses to -40 mV preceded by a 80 ms prepulse to -120 mV from a holding potential of -80 mV, amounted to 415 ± 357 pA ($n = 46$) under control conditions. That evoked by 0 mV pulses was 4.7 ± 1.3 nA ($n = 37$). The T-type barium currents were reversibly reduced by BAB in a concentration dependent way. At the concentration of 200 μ M, barium currents at -40 mV were diminished by 52 ± 8



% (n=6) (Fig. 1C). After washout the currents completely recovered to 96 ± 10 % of the control amplitude. In Figure 1D, the concentration-response relation is shown for the peak of the T-type barium current. This relation was described using the Hill equation, resulting in an IC_{50} of 178 ± 21 μ M and a Hill coefficient of 1.5 ± 0.3 (n=40). This IC_{50} is similar to that found for the N-type barium current evoked at 0 mV (~ 220 μ M (Beekwilder et al., 2005)).

Effect of BAB on the T-type steady-state properties

A hyperpolarizing shift in the inactivation curve has been shown to be a possible current reducing mechanism of action of BAB for sodium channels (Van den Berg et al., 1995; Van den Berg et al., 1996). The typical inactivating time course of the T-type current makes this current an excellent model to look at the effects of BAB on inactivation of calcium channels. For that reason, we measured the steady-state inactivation of the T-type barium current by using a test pulse to -40 mV after applying a 350 ms prepulse to varying potentials from the holding potential -80 mV. The interval between the test pulses was 15 s. This was performed both in the absence and presence of 200 μ M BAB. Plotting the relative current as a function of prepulse voltage resulted in the inactivation curves shown in Fig. 2A. A Boltzmann equation fitted to the data of individual cells yielded midpoint potentials of the inactivation curves under control conditions with a mean value of -52 ± 4 mV (n=5). Application of 200 μ M BAB reduced the currents to less than 50% and induced a small but significant shift of the midpoint to -55 ± 4 mV ($P=0.001$), which could be reversed by washout towards -53 ± 3 mV ($P=0.002$). The normalized voltage-dependent inactivation curves are shown in Fig. 2B. BAB induced a shift of the midpoint of -2.8 ± 0.8 mV, which was not accompanied by a significant change in slope factor, with values of 3.6 ± 0.3 , 3.4 ± 0.4 and 3.5 ± 0.2 mV for control, BAB and washout, respectively. It is clear from Fig. 2A,B that the small BAB-induced hyperpolarizing shift of the steady-state inactivation curve of T-type calcium channels is not responsible for the current reduction observed at the test pulse of -40 mV.

The T-type activation curve can only be obtained in a limited range of voltages due to the overlap with the activation curves of the HVA calcium channels at the more depolarized potentials. In Fig. 2C currents are shown elicited by depolarizing steps to various potentials ranging from -75 mV to -40 mV from a holding potential of -80 mV. This voltage range was limited to these values to

only activate the T-type currents. The resulting peak values were converted to conductance assuming a linear relation between current and driving force with a reversal potential of +50 mV. Subsequently, the conductance-voltage relations for the individual cells were fitted with a Boltzmann equation, which also described the activation curve for the higher range of potentials by extrapolation. The resulting values were averaged to obtain the mean activation curve, which showed no significant shift of the midpoint potential with -41 ± 5 mV for control and -41 ± 3 mV in the presence of 200 μ M BAB ($n = 9$). Nor was there a difference in slope factor with 4.5 ± 0.9 mV and 4.3 ± 0.8 mV for control and BAB, respectively.

In conclusion, BAB caused an overall inhibition of T-type currents, but the steady-state properties of the currents were not or hardly affected. Only the midpoint potential of the inactivation curve was slightly shifted in the hyperpolarizing direction.

Effects of BAB on T-type current kinetics

The current during a maintained depolarizing step to -40 mV from a holding potential of -80 mV for control and with BAB is shown in Fig. 3A. Scaling of the currents to the control peak value showed an accelerating effect of 200 μ M BAB on the currents (Fig. 3B). This acceleration could well be quantified by describing the T-type current traces with a m^2h Hodgkin-Huxley model (Tarasenko et al., 1998), (see methods). The time constant of the activation gate (τ_m) reduced significantly in the presence of BAB from 11.3 ± 2.5 ms to 8.7 ± 3.0 ms ($P < 0.001$, $n = 9$). The inactivation gate (τ_h) accelerated as well with time constants for control and BAB of 91 ± 52 ms and 40 ± 14 ms, respectively ($P = 0.007$, $n = 9$).

Upon repolarization to -80 mV, after a 15 ms pulse to -40 mV, the tail current was measured, representing the deactivation of T-type channels (Fig. 3C,D). In control solution the tail currents decayed with a time constant of 1.70 ± 0.23 ms ($n=5$). Application of 200 μ M BAB significantly reduced this to a time constant of 1.17 ± 0.23 ms ($P < 0.001$). This effect was completely reversible with a time constant of 1.84 ± 0.36 ms ($P < 0.001$) following washout.

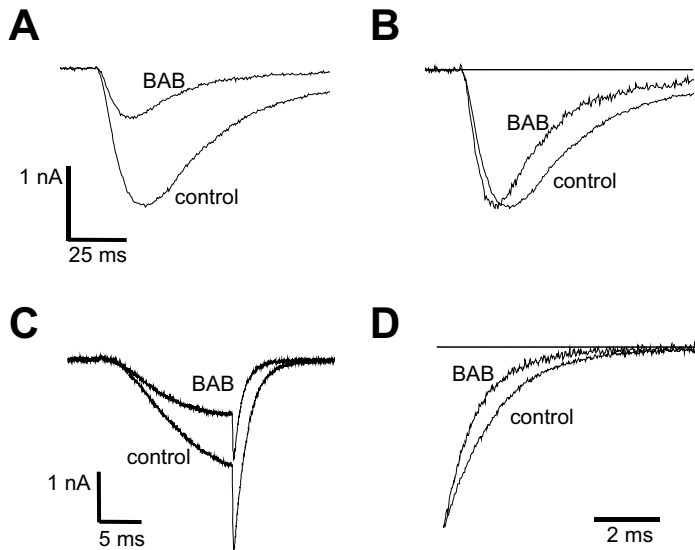


Figure 3. Effects of BAB on T-type current kinetics. (A). Activation and inactivation time course of T-type current records upon a step to -40 mV from a holding potential of -80 mV. Recordings for control and $200 \mu\text{M}$ BAB are superimposed. (B). Currents from 'A' normalized to the control peak value. (C). Barium currents elicited by a 15 ms voltage step to -40 mV from a holding of -80 mV. Recordings for control and $200 \mu\text{M}$ BAB are superimposed. (D). Tail currents from 'C' normalized to their own maximal values.

In conclusion, besides inhibiting T-type currents, BAB also accelerates activation, inactivation and deactivation kinetics of this current.

DISCUSSION

In the present study we specifically determined and analysed the effect of the local anesthetic butamben (BAB) on native low-voltage activated (T-type) calcium channels in the smaller mouse sensory neurons including the nociceptive neurons. BAB reduced the peak currents of the T-type barium current with an IC_{50} of $\sim 200 \mu\text{M}$ and accelerated the kinetics of activation, deactivation and inactivation of this current. These effects of BAB on T-type current are very similar to those on high-voltage activated N-type current (Beekwilder et al., 2005).

Possible mechanism of T-type current inhibition by BAB

In the Hodgkin-Huxley model describing ion channel behavior, altered kinetics are typically reflected in a change of the midpoint potential and slope factor of the steady-state activation and inactivation curves. However, the BAB-induced accelerating effects on T-type current kinetics were not or only weakly accompanied by changes in these parameters. This would imply that the voltage dependent rates of gate opening and closing are roughly proportionally increased. In this interpretation the current inhibition by BAB cannot be fully explained by the observed kinetic changes, because the maximal currents are reduced. Therefore, we consider other than purely kinetic Hodgkin-Huxley mechanisms. The faster deactivation with BAB argues against a classical open-channel block, since that type of block is rather accompanied by slowed deactivation (see Snyders et al., 1992). The described effects on T-type currents are also similar to what was found for Kv1.1 potassium channels (Beekwilder et al., 2003). BAB accelerated both activation and deactivation kinetics without shifting the midpoint potential of activation for Kv1.1 current. In addition there was also an accelerated current decay or inactivation of these currents. These results were explained by an allosteric mechanism with BAB biasing the channels towards nonconducting channel states. Vedantham and Cannon (1999) hypothesized a preferential binding to intermediate closed states, causing increased inactivation in those states and a hyperpolarizing shift of the inactivation curve. Though they studied lidocaine effects, others have found confirming results for benzocaine, which is structurally very similar to BAB (Wang et al., 2004). However, in order to come to definitive conclusions on the underlying mechanisms more experiments would be needed.

The role of T-type currents in pain suppression.

The T-type calcium current seems to be a common neuronal process for mediating excitability. However its biophysical properties determine that it can have complex and paradoxical roles. The activation of the current in the low voltage range has a depolarizing effect, leading to a faster recruitment of sodium channels and therefore to the firing of an action potential. Raman and Bean (1999) showed in Purkinje neurons that blocking the T-type current using mibefadril resulted in a 30 % slowing of the firing rate, which indicated that the

presence of T-type current enhances excitability. McCallum et al. (2003) however, showed an increased excitability in sensory neurons as a result of a T-type current blockade, which means that the T-type presence would be responsible for less excitability. Here the authors suggested that the relatively slow deactivation of the T-type current results in prolonged calcium entry at the end of the action potential. Taking this calcium influx away would lead to higher excitability. The apparent discrepancy between these studies can be explained by the different actions of the T-type current on an action potential depending on the timing of the peak T-type current during this action potential and on the presence of other types of ion channels. The depolarizing action of the T-type current is enhancing the firing rate if it coincides with the uprise of the action potential, yet it inhibits the firing rate if it does so during the repolarizing phase, for example by activating hyperpolarizing calcium activated K channels.

It should also be considered that the T-type current may be carried by three different α -subunits, each with a specific expression pattern in the body. These three pore-forming subunit isotypes contribute differently to neuronal excitability through their different biophysical properties (Chemin et al., 2002). It is this variety of actions that makes it difficult to predict the role of inhibiting T-type calcium currents by BAB in its pain suppressing actions. This is also illustrated by several other studies. Modifying the T-type currents in vivo has shown that T-type currents are involved in pain signalling. Agents that selectively enhance T-type currents result in exaggerated thermal and mechanical nociception, whereas T-type current reducing agents do the opposite (Todorovic et al., 2001). Moreover, suppressing $Ca_v3.2$ (α_{1H}) T-type current, which are expressed in DRG neurons, using the mRNA antisense technique results in antinociceptive, anti-hyperalgesic and anti-allodynic effects (Bourinet et al., 2005). These studies suggest an enhancing nociceptive role for T-type currents. Apparent contradictory results have been found in mice lacking the $Ca_v3.1$ (α_{1G}) gene (Kim et al., 2003), with the absence of T-type currents in the thalamic neurons resulting in hyperalgesia, suggesting an anti-nociceptive role for central T-type currents. This might indicate that T-type currents have different roles depending on where they are located along the pain pathway. The ultralong duration of the pain suppression seems due to a slow steady release of butamben from the suspension in the confined epidural space (grouls?). Hence, the calcium channels present in this space will be subjected to butamben

continuously. Although it is unlikely that inhibiting the T-type calcium current in sensory neurons can alone explain the described pain suppressing effects of epidural BAB, either directly or via the interplay with other BAB affected channels (K_v , and Na_v), BAB effects on T-type calcium channels are likely to play a role, if T-type channels are expressed by dorsal root fibers. However, in the light of the above discussion of the role of T-type channels in pain signal generation in the peripheral nerve endings, the present results do implicate inhibition of T-type channels in BAB containing topical skin applications.

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