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

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CHAPTER 5

THE LOCAL ANESTHETIC BUTAMBEN INHIBITS TOTAL AND L-TYPE BARIUM CURRENTS IN PC12 CELLS.

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ABSTRACT

Background: Butamben or *n*-Butyl-*p*-Aminobenzoate is a long acting experimental local anesthetic for the treatment of chronic pain when given as an epidural suspension. We have investigated whether Cav1.2/L-type calcium channels may be a target of this butamben action.

Methods: The effect of butamben on these channels was studied in undifferentiated rat PC12-cells with the whole-cell patch-clamp technique in voltage-clamp. Ba^{2+} ions were used as the charge carriers in the calcium channel currents, while K^+ currents were removed by using K^+ free solutions.

Results: Butamben 500 μM reversibly suppressed the total whole-cell barium current by 90 ± 3% (n=15), while 10 μM nifedipine suppressed this barium current by 75 ± 7% (n=6). Pre-exposure to butamben followed by wash-out lowered the inhibition by nifidepine to 47 ± 5 % (n=10). These suppressive effects were not due to the measurement procedure and the drug vehicles in the solutions (<0.1% ethanol; n=6). Conclusions: Butamben inhibits the total barium current through expressed calcium channel types in PC12 cells, including Cav1.2/L-type channels. Because Cav1.2 channels may also occur in human nociceptive C fibers, this result allows the possibility that these L-type channels are involved in the analgesic action of butamben.

INTRODUCTION

Terminal cancer patients often suffer from severe pain due to tissue damage caused by either the primary tumor or metastasis. Palliation can be achieved by opioids or, if this does not adequately alleviate the pain, sometimes by ablating sensory nerves. These treatments may cause severe side effects, among which motor dysfunction is the most prominent one. A current experimental approach to chronic pain treatment is the epidural administration of an aqueous suspension of the local anesthetic *n*-butyl-*p*-aminobenzoate, also known as butamben (Shulman, 1987; Korsten et al., 1991). The suspension of butamben applied to the spinal dura results in a long lasting (median 29 days) relief from pain, without impairing motor function or other sensory functions. How butamben produces this extraordinary effect is still largely unresolved.

The butamben molecule is an aminobenzoate ester-linked to a butyl group. Its structure is similar to that of other ester-linked local anesthetics such as benzocaine and procaine, which block sodium channels involved in impulse generation and transmission in neurons (Butterworth and Strichartz, 1990; Hille, 2001). The effects of butamben on sodium currents have previously been studied in small DRG neurons (Van den Berg et al., 1995), which are believed to include the cell bodies of nociceptive fibers (Van den Berg et al., 1996). Butamben (100 μM) had a diverse effect on the various types of sodium channels, ranging from nearly completely blocking fast sodium currents to having no effect on slow sodium currents. The inhibition of DRG fast sodium currents resulted in reduced excitability of DRG neurons, which is likely to contribute to the butamben anesthesia. However, the blocking effect of butamben on the fast sodium currents does not seem to be the only mechanism of butamben analgesia (Butterworth and Strichartz, 1990).

Inward current through calcium channels also plays an important role in action potential generation in sensory neurons (Scroggs and Fox, 1992) and possibly also in human impulse transmission in C-type nocifibers (Quasthoff et al., 1995). Recently, two types of calcium channels that are expressed in neonatal mouse dorsal root ganglion (DRG) neurons, N-type and T-type, were shown to be suppressed by butamben (Beekwilder et al., 2005; Beekwilder et al., 2006) with

a 50% inhibiting concentration of ~200 μM, similar to that for inhibition by butamben of the total calcium or barium current through all the calcium channels . These mouse DRG neurons also express L-type calcium channels (subtype Cav1.2), but in a proportion too small (7 ± 6 %, n=7; unpublished observations) to study with our whole-cell current recording technique. In small adult rat DRG neurons, however, voltage gated L-type calcium channels seem to constitute a significant portion of calcium currents (Scroggs and Fox, 1992). Hence, it is interesting to explore the effect of butamben on L-type calcium currents.

Therefore, we addressed the question whether butamben inhibits the Cav1.2/L-type current component of whole-cell barium currents through calcium channels. To this end, the patch-clamp technique in whole-cell voltage-clamp configuration was applied to undifferentiated PC12 (pheochromocytoma) cells. These are rat adrenal medullar chromaffin tumor cells that express various types of calcium channels, with a relatively strong expression of the cardiac L-type (α1C; Avidor et al., 1994; Liu et al., 1996), denoted as Cav1.2 in modern terminology (Hille, 2001). By making use of nifedipine, a specific L-type calcium channel blocker (Hille, 2001), we show that butamben, besides blocking the total barium current through calcium channels, at least partly blocks the L-type barium current component in PC12 cells. In the discussion, we consider the implications of the present results for the analgesic action of butamben.

METHODS

PC12 cell culture

PC12 cells from the Hubrecht Laboratory (Utrecht, The Netherlands) were maintained in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated horse serum, 5% fetal calf serum (both sera from Invitrogen, Breda, the Netherlands), 100 IU/mLpenicillin, and 100 μg/mL streptomycin (both from Sigma-Aldrich, Zwijndrecht, the Netherlands). After

cells were grown in a poly-L-lysine (MW 70,000-150,000 D, Sigma-Aldrich) coated culture flask for 7 days and had formed a nearly confluent monolayer, they were dissociated with Versene (Invitrogen) and plated on poly-L-lysine coated cover slips, after which they were grown in a culture dish in a humidified 5% CO_2 incubator at 37˚C to obtain undifferentiated PC12 cells (Avidor et al., 1994). Experiments were conducted 4-7 days after plating.

Whole-cell recording

The patch-clamp experiments were carried out at room temperature (~23 ^{o}C). A glass coverslip was mounted in a chamber on the stage of an inverted microscope (Zeiss Axiovert 35). Patch pipettes were fabricated from borosilicate glass (Harvard Apparatus, Edenbridge, Kent, UK) and were giga-sealed to the cells in a microbath (75 µL) continuously perfused with a standard extracellular solution (ECS), containing (in mM) 125 NaCl, 5.5 KCl, 0.8 MgCl2, 2 CaCl2, 10 HEPES/NaOH (pH 7.3), 21.8 glucose and 36.5 sucrose, which is similar to the solution used by (Westerink et al., 2000) for PC12-cells. The pipette was filled with a CsCl containing intracellular solution (CsICS), consisting of 130 CsCl, 1 CaCl2, 10 HEPES/CsOH (pH 7.2), 10 EGTA, 5 MgATP and 0.5 TrisGTP. Pipette resistance measured in ECS was 4.2 ± 0.1 MΩ (mean ± SEM, n = 23). Flattened adhered polygonal cells were preferred over phase-bright spherical cells, because they exhibited larger calcium channel currents (Janigro et al., 1989). Seal resistances were 1.8 ± 0.2 GΩ (n=18). After establishment of the whole-cell configuration, the microbath was perfused with a solution containing barium (BaECS), consisting of 140 NaCl, 5 CsCl, 2 MgCl2, 10 BaCl2 and 10 HEPES/NaOH (pH 7.3). The combined use of BaECS and CsICS enhanced the current through calcium channels and fully removed potassium currents (cf. Fig. 1). Occasional (in <5% of the cells) inward sodium-like currents (Garber et al., 1989) were small and so fast (~3-ms duration) that they did not interfere with our measurements of the slower barium currents. Butamben (OPG Farma, Utrecht, The Netherlands) was added to the BaECS in a concentration of 500 µM, from a stock of 500 mM butamben in ethanol. Nifedipine (Sigma-Aldrich) was used to identify currents through L-type channels. It was added to the BaECS in a concentration of 10 µM, from a stock of 10 mM nifedipine in ethanol. This concentration is close to that for maximal and specific inhibition of Ca1.2/L-type channels under our measurement conditions, i.e. at a holding potential of -80 mV (Hille, 2001).

Both for the butamben and nifedipine solution the concentration of ethanol never exceeded 0.1%.

A PC running Clampex 8 (Axon Instruments, Foster City, CA) and a List EPC 7 amplifier provided voltage protocols. The membrane currents were filtered at 3 kHz. The PC12 cell currents were leak subtracted using the P/4 method. The single exponential capacitive transients revealed the absence of electrical coupling between cells, even when they were visibly in contact. The membrane capacitance of the cells derived from these transients was 24.4 $\pm$ 3.1 pF (n=27). The series resistance was 7.2 $\pm$ 0.6 MΩ (n=9) and was not compensated because of the small size of the recorded currents. Data are presented as mean ± SEM for n cells. Means are compared using paired or independent t-tests with the level of significance (p) chosen as 0.05.

RESULTS

Upon depolarization, the selected PC12 cells exhibited a small inward calcium current in ECS, consisting of currents through various types of calcium channels (Garber et al., 1989; Janigro et al., 1989; Liu et al., 1996). To enhance the current flowing through the calcium channels, barium ions were used as charge carriers (instead of calcium ions) by applying a barium solution (BaECS) to the cells. The cell membrane was held at a voltage of –80 mV and was then step-depolarized to +10 mV for 60 ms at regular intervals of 15 s until the inward barium current reached its maximal increase (within 4 min, including the arrival-delay of Ba-ECS through the perfusion tubing). Figure 1A shows the inward calcium current of a PC12 cell at 10 mV, as well as the gradual increase in the current at that test potential upon infusion of the barium containing solution into the bath. The calcium current records in ECS showed a variable composition of a faster and slower inactivating current (see example in Fig. 1A). A current-voltage (I-V) relationship of the barium current was created by applying test potentials between –60 and +40 mV to the PC12 cells at 15 s intervals and by measuring

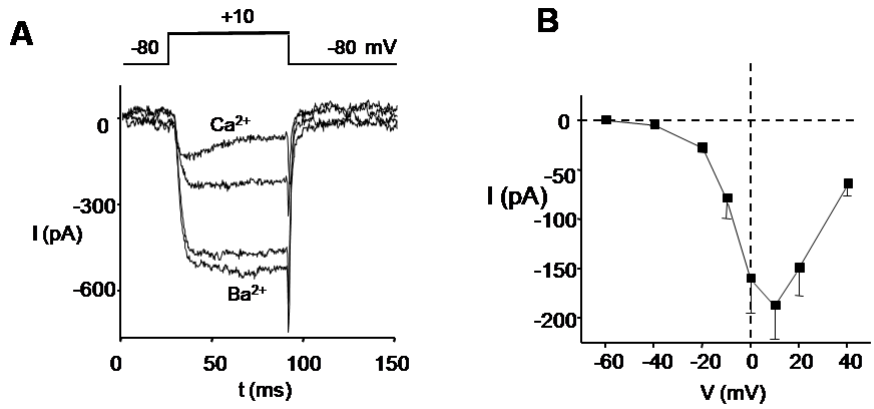

Figure 1. (*A*) Progress in calcium channel current increase upon exchanging the calcium containing bath solution (ECS) for a barium containing solution. Current records were evoked by voltage steps from –80 to +10 mV with 15-s intervals, of which here every 4th response is shown. The upper record is taken in the calcium containing ECS, just before infusing the barium solution. The lowest record is an early steady-state record in the barium containing solution. Note also the absence of the fast inactivation time course in the increased barium current. (*B*) Mean I-V relationship gained by applying depolarizing voltages from a holding potential of –80 mV, after which the barium current's maximum was measured which was then plotted against the voltage at which it was elicited (mean ± SEM, n=8).

the maximal barium current at each test potential (Fig. 1B). From about –40 mV upwards the barium current increased in amplitude until it reached its maximum at +10 mV (~-200pA), after which it declined and reversed at potentials extrapolated to >40 mV. To establish whether butamben inhibited the barium current, 500 µM butamben was applied to the cells. This concentration of butamben blocked 90 ± 3% of the control barium current (P <0.05, n=15) (Figure *2A*), which level was reached within 4 min, including the butamben arrival delay. This inhibitory effect was largely reversible to 76 ± 6 % (n=8) after ~ 5 minutes wash-out. Now that the blocking effect of butamben on the total barium current through calcium channels expressed in PC12 cells was established, 10 µM nifedipine was applied to the cells to prove that at least part of the calcium channels in the PC12 cell membranes was of the L-type. 10 µM nifedipine blocked 75 ± 7% of the control barium current (P < 0.05, n=6) (Fig. 2B) within about 3 min. This effect was partly reversible to 47 ± 7 % (n=6) of the initial

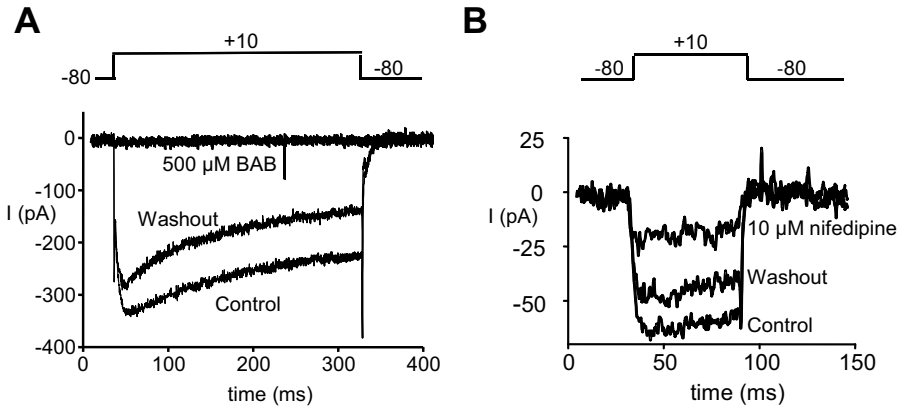

Figure 2. (A) Addition of 500 μM butamben (BAB) resulted in an almost complete inhibition of the barium current. Note also the increased inactivation rate in the washout record. (*B*) Addition of 10 μM nifedipine resulted in an inhibition of a large part of the barium current. Note the difference in time scales in A and B and Fig. 1, indicating variability in calcium channel expression between cells.

control current, after ~5 min (Fig. 2B). Assuming that the nifedipine only affected the L-type current (Hille, 2001), we conclude that at least three quarters of the barium currents generated by the PC12 of the L-type and that since 500 μM butamben blocked 90% of that current, L-type calcium channels are at least partly blocked by butamben.

To gain further support for this conclusion, an experiment was done in which the effects of butamben and nifidepine were determined on the same cell. After an initial barium current control period, a PC12 cell was first exposed to 500 μM butamben for 4 min, after which buamben was washed out for 5.5 min and 10 μM nifedipine was added for 4 min. Finally, nifedipine was washed out for 3.5 min to check for reversibility of the nifedipine effect. During the application and washing out of the drugs, the cells were step-depolarized from a holding potential of –80 mV to 10 mV with 15-s intervals to elicit the barium current and explore the effect of the drugs on the current. Figure 3A shows example records, while the mean results of 10 of these experiments are shown in Figure 3B. Butamben 500 μM caused a 93 ± 2% inhibition of the inward barium current ($P < 0.05$, n=10), reproducing the above results. When butamben was washed out, 74 ± 5% of the original current was regained ($P < 0.05$, n=10), also corresponding

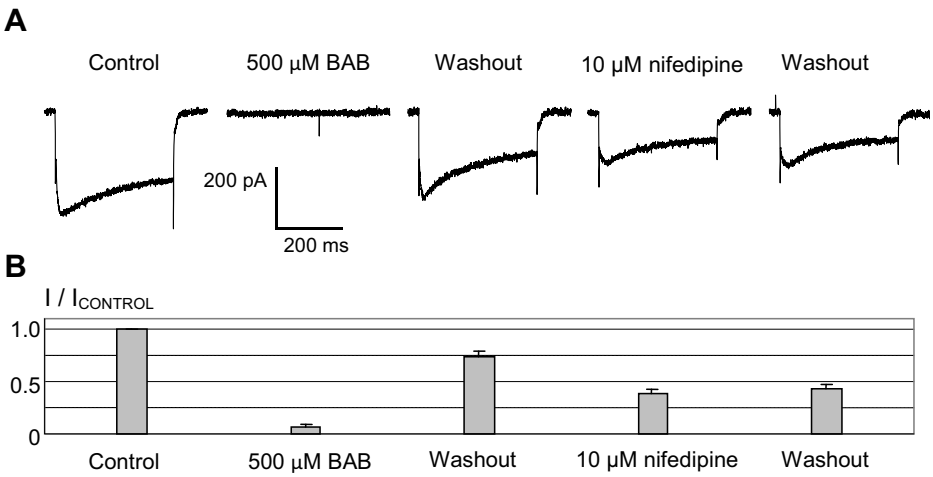

Figure 3. (*A*) The effect of butamben (BAB) and nifedipine on barium currents elicited in one and the same cell by a voltage step to +10 mV from a holding potential of –80 mV. Barium currents are given for the control condition, after addition of 500 µM butamben, after washout of butamben, after addition of 10 µM nifedipine and after washout of nifedipine, respectively. (*B*) Results of 10 experiments in which the cells were exposed to 500 µM butamben as well as to 10 µM nifedipine, according to the protocol in A. Normalized mean peak barium currents ± SEM are given for each condition.

to the above results. When 10 µM nifedipine was then applied to the cells, we found an inhibition of 47 ± 5 % (n=10) of the preceding butamben wash-out current peak, corresponding to a remaining 38 ± 4% of the original current ($P < 0.05$, n=10). This inhibition is smaller than the fresh exposure inhibition of 75% described above. The final barium current amplitude after washout of nifedipine was 43 ± 4% of the original current amplitude ($P > 0.05$ washout versus nifedipine, n=7), which corresponds to a recovery of ~57% of the preceding butamben wash-out peak current.

Since the inward barium current did not completely recover after washout of butamben and nifedipine, the possibility remained that part of the inhibition of the barium current seen during and after the administration of butamben and nifedipine was caused by rundown of the barium current. Therefore, we carried out a vehicle/rundown control experiment, in which the same drug application protocol was used as in the experiment described above. After an initial Ba

wash-in period of ~4 min, we exposed the cells, instead to butamben and nifedipine, to 0.1% vehicle (ethanol), which also allowed us to check whether the inhibiting effects of butamben and nifedipine were not due to ethanol. Figure 4 shows only a slight decline of the normalized peak barium-current amplitude during the course of the experiment. At the end of the experiment (after ~18 min), 85 ± 3% (n=6) of the original barium current remained. The conclusions that can be drawn from this experiment are that the inhibiting effects seen during the administrations of butamben and nifedipine were not caused by the vehicle (0.1% ethanol) and that the rundown of the barium current may only explain a small percentage (≤15%) of the incomplete recovery after butamben and nifedipine exposure.

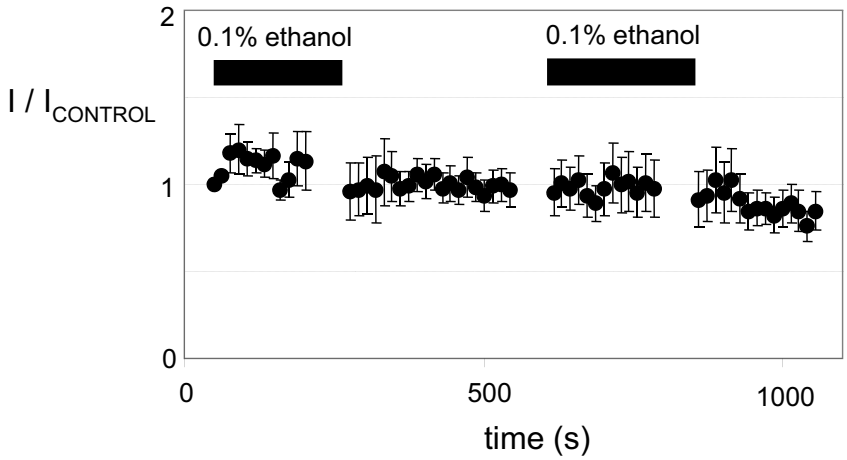

Figure 4. The rundown/vehicle control experiment. Peak barium currents were measured upon depolarizing steps to +10 mV from a holding potential of –80 mV, applied with 15-s intervals. Black horizontal bars indicate the addition of vehicle (0.1% ethanol) according to the protocol in Fig. 3. Peak currents were normalized by dividing these currents by the peak current at t = 0 s. All experiments were preceded by a 4-min in-wash period of BaECS (not shown). Mean normalized peak currents ± SEM are plotted for 6 cells. Empty spaces in the plot are interruptions for voltage-clamp protocols to determine I-V curves.

DISCUSSION

In the present study we examined the inhibiting effect of butamben on calcium channels, including Cav1.2/L-type channels, in PC12 cells. These channels are expressed in small rat DRG neurons and therefore may contribute to pain signal transmission (Scroggs and Fox, 1992). We rather used undifferentiated PC-12 cells than DRG-neurons, because L-type calcium channels constitute in these cells a significant part of the expressed calcium channels (Janigro et al., 1989; Avidor et al., 1994), providing about 75 % of the peak barium current in our study. We found that the clinically relevant concentration of 500 µM butamben (close to the maximum solubility concentration of ~700 µM in butamben suspensions, see chemical 1504 in The Merck Index , 1989) blocked ~90 % of the total peak barium current that was mediated by the various types of calcium channels (besides L- probably also N- and T-type) expressed in PC12 cells (Garber et al., 1989; Janigro et al., 1989; Avidor et al., 1994). This result is consistent with our earlier studies (Beekwilder et al., 2005; Beekwilder et al., 2006) on sensory neurons, which showed that butamben inhibits the total barium or calcium current of the smaller neonatal mouse dorsal-root ganglion neurons and specifically the barium currents through N- and T-type channels by 80-90%. Total Kv and isolated Kv1.1 currents were also inhibited for ~80% by 500 µM butamben (Beekwilder et al., 2003). The concentration response curves in all these cases had an IC50 around 200µM (range 177-238) and a Hill coefficient of ~1.5 (range 1.1-1.8). We expect therefore that Cav1.2 currents have a similar concentration response curve for butamben.

In our preparations of sensory neurons the L-type currents were too small to be of use for the study of the effect of butamben. We proved here that at least part of the L-type calcium channels in PC12 cells was inhibited by butamben, because the inhibition of the total barium current (~90%) was clearly larger than the percentage of L-type current, which was 75 or 47%, depending on the used inhibition protocol. Our results also show that the inhibiting effect of butamben on calcium channels is not cell type or species dependent.

It is noteworthy that in the longer protocol, where first butamben was applied to the cells and washed out, and then nifedipine was administered, nifedipine

seemed to inhibit a smaller portion of the barium current (47%) than in the protocol in which nifedipine was the first-exposure drug (75%). One reason may be that the different calcium channel subtype components have differences in run-down and/or run-up time courses, changing the proportions of these components of the total barium current at the observed run-down of ~15% over 18 min. Another possibility is that washout of the butamben effect was not complete, and that remaining butamben molecules interfere with nifedipine binding. Nevertheless, it was proven that butamben inhibits L-type calcium channels. Through which mechanism butamben reaches this effect is still unknown. One mechanism might be that butamben causes a relative acceleration of deactivation and inactivation kinetics, which would make it more difficult for the channel to open and stay open in the presence of butamben. This would be consistent with a BAB induced increase in deactivation and inactivation rate of other calcium channels (N- and T-type) and of Kv1.1 channels as observed by Beekwilder et al. (2003; 2005; 2006). In this respect it is worth mentioning that incompletely recovered barium currents after butamben wash-out often showed an increased inactivation rate (Figs. 2A and 3A). Further studies directed at butamben's effect on the gating kinetics of the L-type calcium channel could help clarify butamben's mechanism of action.

The mechanism of butamben analgesia depends on butamben's ability to suppress the generation and/or transmission of action potentials in the neurons that transmit pain signals to the brain. Since L-type calcium channels are possibly present in human nociceptive C fibers (Quasthoff et al., 1995), Butamben's blocking effect on this type of calcium channels might contribute to analgesia when administered epidurally. The present results and those of Beekwilder et al. (2005; 2006) add butamben to the list of local anesthetics inhibiting calcium channels (Sugiyama and Muteki, 1994). They also implicate anesthetic actions of butamben on the peripheral autonomous nervous system. Future studies should be directed at the question how the integrated effects of butamben on the various types of ion channels in DRG neurons, result in analgesia.

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